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APPLICATION NUMBER: *60/533,103*
FILING DATE: *December 29, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/11988*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
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15866 U.S. PTO

EXPRESS MAIL NO. EL852794736US

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a requ st for filing a PROVISIONAL APPLICATION FOR PATENT und r 37 CFR 1.53 (c).

60/533103 122903

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Ashlee V.	Moses	Portland, Oregon

☒ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHODS OF TREATMENT AND DIAGNOSIS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND HIV-RELATED DISEASES

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☒ Specification *Number of Pages* 68

☒ Drawing(s) *Number of Sheets* 6

☒ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☒ Other (specify) Fee Transmittal (+ copy Sequence Listing, paper and CRF (2), Sequence Statement, Postcard indicating receipt

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order for $_____ is enclosed to cover the filing fees.

☒ The Commissioner is hereby authorized to charge filing fees to Deposit Account Number: 04-0258

☒ The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account Number: 04-0258

☐ Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: NIH/NIAID 1R41 AI055218-01.

Respectfully submitted,			
SIGNATURE		DATE	29 Dec. 2003
TYPED or PRINTED NAME	Barry L. Davison, Ph.D., J.D.	REGISTRATION NO. *(if appropriate)*	47,309
TELEPHONE	206-628-7621	DOCKET NUMBER:	49321-106

SEA 1447758v1 49321-106
Seattle

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	Moses
Examiner Name	
Art Unit	
Attorney Docket No.	49321-106

METHOD OF PAYMENT (*check all that apply*)

☐ Check ☐ Credit card ☐ Money Order ☐ None

☒ Deposit Account:

Deposit Account Number: 04-0258

Deposit Account Name: Davis Wright Tremaine LLP

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FEE CALCULATION

1. **BASIC FILING FEE**

Large Entity Fee Code	Large Entity Fee($)	Small Entity Fee Code	Small Entity Fee($)	Fee Description	Fee Paid
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1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80
				SUBTOTAL (1)	($)80

2. **EXTRA CLAIM FEES**

			Extra Claims		Fee from below		Fee Paid
Total Claims		- 20** =		x		=	
Independent Claims		- 3** =		x		=	
Multiple Dependent						=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** *Reissue independent claims over original patent*
1205	18	2205	9	** *Reissue claims in excess of 20 and over original patent*

SUBTOTAL (2) ($)0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. **ADDITIONAL FEES**

Large Entity Fee Code	Large Entity Fee ($)	Small Fee Code	Small Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive – unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Petitions related to provisional applications	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) ____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)0

SUBMITTED BY			(Complete (*if applicable*))		
Name (Print Type)	Barry L. Davison, Ph.D., J.D.	*Registration No. (Attorney/Agent)*	47,309	Telephone	206-628-7621
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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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	49321-106	Type a plus sign (+) inside this box →	+

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Jay	Nelson	Tualatin, Oregon
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Laura	Jelinek	Tigard, Oregon

Number 2 of 2

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MAILING CERTIFICATE

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as Express Mail Label No. EL852794736 US in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on Dec. 29, 2003 by Kay Bulen Kay Bulen

Date Signature (print name)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Moses et al.

Filed: December 29, 2003

Serial No.: to be assigned

For: METHODS OF TREATMENT AND DIAGNOSIS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND HIV-RELATED DISEASES

Docket No.: 49321-106

Date: December 26, 2003

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT UNDER 37 C.F.R. §1.821(f)

Sir:

I hereby state that the content of the paper and computer-readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. §1.821, are the same.

Respectfully submitted,

Barry L. Davison, Ph.D., J.D.
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SEA 1447793v1 49321-106

Docket Number: 49321-106
Express Mail Number: EL852794736US

METHODS OF TREATMENT AND DIAGNOSIS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND HIV-RELATED DISEASES

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This work was partially funded by NIH/NIAID grant number 1R41 AIo55218-01, and the United States government has, therefore, certain rights to the present invention.

FIELD OF THE INVENTION

The present invention relates to the identification and use, including therapeutic use, of modulators of human immunodeficiency virus (HIV)-induced cellular gene expression. Preferred modulators are inhibitors capable of reducing the expression of HIV-induced genes, reducing or preventing the expression of mRNA from HIV-induced genes, or reducing the biological activity of corresponding HIV-induced cellular gene products. The invention provides therapeutic methods, diagnostic methods and compositions useful for the treatment of HIV-related disorders and disease. Particular embodiments also provide drug candidate screening assays. The present invention uses nucleic acid microarrays and gene expression profiling, along with antisense oligonucleotide and RNA interference (siRNA) methods to identify and validate, respectively, therapeutically useful gene targets that are regulated upon HIV infection and replication.

BACKGROUND

The AIDS epidemic continues to spread, and urgent efforts are required to stem this global health crisis. While modern antiretroviral drugs have enabled many HIV-positive individuals to live longer and delay progression to AIDS, these drugs do not ultimately cure infection, and long term use is associated with toxicity and the emergence of drug resistant strains. Cost and delivery issues also make such therapies prohibitive in much of the developing world, and the development of an effective vaccine has, at least to date, proved elusive.

Significantly, the high mutation rate of HIV enables immune escape, as well establishment of eradication-resistant latent reservoirs, favoring persistence of the virus despite immunization or antiretroviral therapy. Another reason for the success of HIV as an intracellular pathogen is its remarkable ability to exploit the molecular machinery of the host cell to facilitate persistence, replication and spread of the virus. The virus has likely evolved mechanisms to modulate expression levels of these cellular co-factors to promote its life cycle.

Therefore, there is a strong need in the art to disrupt key interactions between HIV and the host cell facilitators of infection and viral replication by counteracting the viral modulation of those genes. Central to the success of this approach however is the initial *identification* of these cellular co-factors and clarification, and further, *validation*, of their role in the virus life cycle.

Limited prior art identification. A recent paper describes the identification of an anti-HIV cellular factor that is thought to be the human cell target of the HIV-vif protein (Sheehy et al. *Nature* 418:646-650, 2002). This protein, named CEM15, possesses significant amino acid similarity to the mRNA-editing cytidine deaminase apobec-1. Although wild type levels of vif are sufficient to overcome the action of CEM15, this factor renders vif-defective virions non-infectious, and thus expression of a vif-resistant form of this protein may be of therapeutic interest. Significantly, however, the studies of Sheehy et al were limited by their utilization of a PCR-based cDNA subtraction strategy to identify CEM15. Additionally, there is a need for further validation of such potential therapeutic targets.

There is a need in the art for additional methods and studies to distinguish, from among those HIV-regulated cellular gene sequences, those actually required for HIV-induced proliferative and phenotypic/developmental changes and which could therefore provide *validated* intervention targets for the inhibition of HIV-induced cellular phenomena and the treatment of HIV-related diseases and hyperproliferative disorders such as cancer. There is a need in the art for compositions and methods to affect such validated targets, and for screening a diagnositic assays premised on such validated HIV-relevant targets.

SUMMARY OF THE INVENTION

Nucleic acid microarray techniques were used in combination with HIV-infected myeloid (*e.g.*, THP-1) and T cell (*e.g.*, MT-2) lines to identify and *validate* cellular genes and pathways useful in modulating virus during the complete replication cycle of Human immunodeficiency virus (HIV-1; *e.g.*, the dual-tropic (X4/R5) isolate 89.6). The present EXAMPLES 1-4 show that modulators of the expression of particular novel validated HIV-induced cellular gene targets are suitable agents for treating HIV and HIV-related cancer and hyperplastic/neoplastic conditions.

The present invention provides modulators of HIV-induced cellular gene expression including, but are not limited to antisense molecules, siRNA agents, ribozymes, antibodies or antibody fragments, proteins or polypeptides as well as small molecules. The inventive modulators are useful for reducing the expression of HIV-induced genes, reducing or preventing the expression of mRNA from HIV-induced genes, or reducing the biological activity of corresponding HIV-induced cellular gene products. Preferably, the inventive modulators are directed to one or more validated HIV-induced gene targets, the expression of which is required, at least to some extent, for HIV infection, replication, and HIV-mediated cellular effects, conditions and diseases.

Particular embodiments of the present invention provide therapeutic methods and compositions for modulation of HIV infection and/or replication comprising use of inventive modulators for inhibition of the expression of HIV-induced cellular genes, reducing or preventing the expression of mRNA from such HIV-induced genes, or reducing the biological activity of corresponding HIV-induced cellular gene products.

Preferred inventive modulators are oligonucleotides, such as antisense molecules or ribozymes, RNA interference (siRNA) methods and agents, for targeting and/or modulating the expression of polynucleotides (*e.g.*, mRNA) comprising HIV-induced gene sequences.

Preferred antisense molecules or the complements thereof comprise at least 10, 15, 17, 20 or 25 consecutive complementary nucleotides of, or hybridize under stringent or highly stringent conditions to at least one of the nucleic acid sequences from the group consisting of SEQ ID

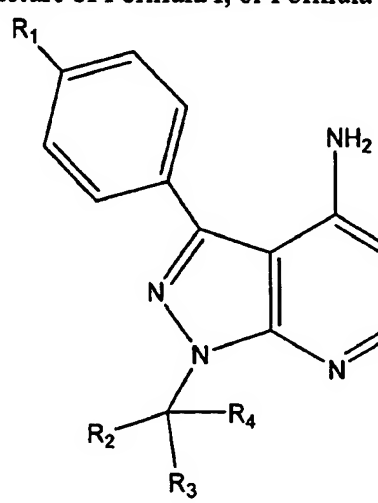
NOS:1, 3, 5, 6, and 8, and complements thereof. Preferably, such antisense molecules are PMO (phosphorodiamidate morpholino oligomers) antisense molecules.

Preferred compositions comprise one or more of such modulators or preferred modulators, along with a pharmaceutically acceptable carrier or diluent.

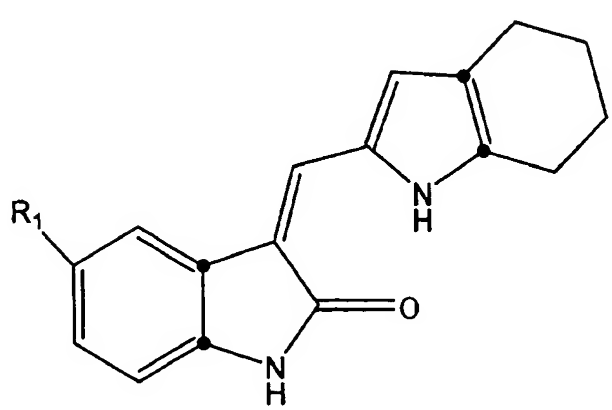
Additional embodiments provide screening assays for compounds useful to modulate HIV infection.

Further embodiments provide a method for inhibiting HIV infection and/or replication comprising administration of an *src* family kinase inhibitor selected from the group consisting of *src* family kinase-specific antisense RNA, *src* family kinase-specific siRNA, and a small molecule inhibitor of a *src* family kinase.

Preferably, the *src* family kinase is c-yes kinase. Preferably, the inhibitor is compound having the structure of Formula I, or Formula II, or salts thereof:



Formula I



Formula II

Preferably, for Formula 1, R_1 is halogen, and R_2, R_3 and R_4 are independently a C1-C3 straight or branched alkyl. Preferably, for Formula II, R_1 is $-SO_2N(CH_3)_2$, or $-SO_2NH_2$. Preferably, the inhibitor is 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Preferably, the inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

Further embodiments provide diagnostic or prognostic assays for HIV infection and/or replication and related conditions and disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the potency of concentrated HIV virus (89.6) stocks using MAGI cells infected with 1 μl, or 0.1 μl of *concentrated* virus (leftmost panel, and center panel, respectively), as compared to 1 μl of *unconcentrated* virus (rightmost panel). MAGI cells are Hela CD4 cells stably transfected with the β-galactosidase gene under the control of the HIV LTR. When MAGI cells are productively infected with HIV, β-galactosidase expression is induced by tat-transactivation and the number of blue cells revealed by staining is a measure of virus titer as can be seen in the left and right panels (MAGI cells infected with 0.1 μl of unconcentrated HIV 89.6 exhibited no sign of infection).

Figure 2A shows, by fluorescence microscopy (left panel), MT-2 cells (human T cell leukemia cell line) efficiently loaded with a FITC-tagged phosphorodiamidate morpholino oligomer (PMO).

Figure 2B shows extensive HIV-induced syncytia in MT-2 cells at 48 hrs PI (post-infection).

Figure 3A shows inhibition curves of HIV replication in HIV-infected THP-1 cells (human myeloid (monocyte/macrophage) cell line derived from an acute monocytic leukemia) by PMOs specific for particular HIV-induced cellular genes as follows: upper filled diamonds correspond to no PMO control; triangles correspond to TNIP; lower filled diamonds correspond to c-YES; dark "X"s correspond to HRH1; light "X"s correspond to NP; filled squares

correspond to HMG20; and vertical lines correspond to AZT control. The HIV-1 P24 ELISA assay monitors HIV p24 gag production (in pg/ml along the y-axis) by PMO-treated HIV-infected THP-1 cells. The x-axis shows time. PMOs corresponding to HMG20, HRH-1, NP and c-YES were particularly effective at reproducibly inhibiting HIV replication.

Figure 3B shows inhibition curves of HIV replication in HIV-infected MT-2 cells (human T cell leukemia cell line) by PMOs specific for particular HIV-induced cellular genes as follows: upper curve filled diamonds correspond to EPEI (ethoxylated polyethylenimine); open squares correspond to HIV only; open triangles correspond to ARF; filled triangles correspond to NP; lower curve filled diamonds correspond to HMG20; "X"s correspond to c-YES; filled squares correspond to HRH-1; and vertical lines correspond to HIV plus AZT control. The HIV-1 P24 ELISA assay monitors HIV p24 gag production (in pg/ml along the y-axis) by PMO-treated HIV-infected MT-2 cells. The x-axis shows time. As in the case of HIV-infected THP-1 cells (Figure 3A), PMOs corresponding to HMG20, HRH-1, NP and c-YES were particularly effective at reproducibly inhibiting HIV replication.

Figure 4 shows inhibition of HIV p24 production in MT-2 cells infected with HIV 89.6 in the continued presence (10 μM) of the src family kinase inhibitor PP2 [4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine]. PP3, and DMSO correspond to inactive analog, and carrier control, respectively. AZT inhibition is also included as a positive control. Time 0 is immediately post-infection, whereas times 1, 2 and 3 correspond to 24, 48, and 72 hrs PI, respectively. The curves are as follows: upper closed diamonds correspond to HIV alone; lower curve closed diamonds correspond to PP2; filled squares correspond to PP3; filled triangles correspond to DMSO; and "X"s correspond to AZT. The HIV-1 P24 ELISA assay monitors HIV p24 gag production by the various treated HIV-infected MT-2 cells.

DETAILED DESCRIPTION OF THE INVENTION

IDENTIFICATION OF HIV-REGULATED GENES AND PATHWAYS, VALIDATION OF SAME AS THERAPEUTIC TARGETS, AND PROVISION OF THERAPEUTIC MODULATORS

Overview

The present invention provides new classes of drugs to combat human immunodeficiency virus (HIV) infection through the identification of novel target cellular genes essential for HIV replication in human cells. Cellular drug targets were identified through gene expression profiling of HIV-infected cells, and validated using target-specific gene silencing techniques with HIV replication assays as the readout.

Recent advances in microarray technology have made possible the analysis of global gene expression patterns in cells in response to viral infection, including HIV infection. However, many of these studies utilized gene expression profiling to examine the consequences of expressing only certain HIV proteins (*e.g.*, Tat and Nef), individually in target cells, and are not as relevant for evaluating the more complex consequences of a dynamic HIV infection.

Significantly, a comprehensive analysis utilizing cell lines to examine the effects of HIV on cellular gene expression has yet to be performed. Only two studies published to date have analyzed acute infection by replication competent HIV (Geiss, G. K. et al., *Virology* 266:8-16, 2000; and Corbeil, J. et al., *Genome Res* 11:1198-204, 2001). Both studies utilized the CEM T cell line and a single strain of HIV, HIV_{LAI}. The study by Geiss *et al* (*supra*) analyzed only 2 time points at 48 and 72 hours post infection and the potential number of genes of interest was constrained by the use of a gene array representing only 1500 genes (a cDNA microarray). The study by Corbeil *et al* (*supra*) was more extensive, examining 6800 genes at 8 time points over a 72-hour period.

The present invention uses high-throughput gene expression profiling on an extensive platform, and gene silencing methods to identify and provide a plurality of 'validated' HIV-induced cellular gene sequences and pathways useful as targets for modulation of HIV-mediated effects and phenotype associated with HIV. Validated gene targets correspond to those HIV-

induced gene sequences the expression of which is required, at least to some extent, for HIV infection and/or replication, or HIV-mediated cellular effects, conditions, diseases and phenotypes. Inventive modulators of validated targets are agents that act by inhibiting the expression of such validated HIV-induced cellular genes, by reducing or preventing the expression of mRNA from such validated HIV-induced genes, or by reducing the biological activity of corresponding HIV-induced cellular gene products. Inventive modulators of HIV-induced cellular gene expression include, but are not limited to antisense molecules, siRNA agents, ribozymes, antibodies or antibody fragments, proteins or polypeptides as well as small molecules.

Applicants have previously used gene expression profiling, and gene silencing methods involving a KSHV-DMVEC model to identify and provide a plurality of 'validated' KSHV (Kaposi's Sarcoma)-induced cellular gene sequences and pathways useful as targets for modulation of KSHV-mediated effects and phenotype associated with KSHV (*see* Moses, A. V. et al., *Ann. N.Y. Acad. Sci* 975:1-12, 2002, incorporated herein by reference).

DEFINITIONS

The term "HIV" refers to the human immunodeficiency virus.

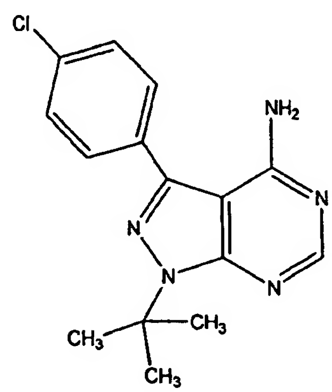
The term "89.6" or "HIV 89.6" refers to isolate 89.6 of HIV.

The term "siRNA" or "RNAi" refers to small interfering RNA as is known in the art (*see e.g.*: U.S. Patent 6,506,559; Milhavet et al., *Pharmacological Reviews* 55:629-648, 2003; and Gitlin et al., *J. Virol.* 77:7159-7165, 2003; incorporated herein by reference).

The term "MT-2 cells" refers cells of the art-recognized human T cell leukemia cell line MT-2.

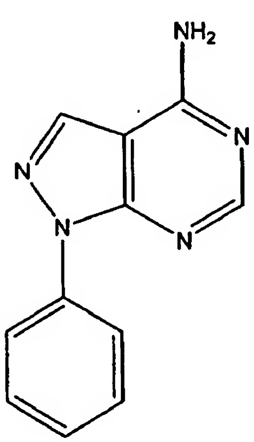
The term "THP-1 cells" refers cells of the art-recognized human human myeloid (monocyte/macrophage) cell line derived from an acute monocytic leukemia.

The term "PP2" (see formula below) refers to 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (*e.g.*, Calbiochem; catalog no. 529573), and physiologically acceptable salts thereof:



PP2

The term "PP3" refers to 4-amino-7-phenylpyrazol[3,4-D]pyrimidine (PP3) (Calbiochem), a negative control for PP2, and salts thereof:



PP3

The term "SU6656" refers to (2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide) (*see* Blake et al., *Mol. Cell. Biol.* 20:9018-9027, Dec. 2000), and physiologically acceptable salts thereof.

The term "SU6657" refers to (2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid amide) (*Id*), and salts thereof.

The term "HMG20B" is used in reference to the gene, cDNA, mRNA or corresponding protein sequences of *homo sapiens* high-mobility group 20B (accession number NM_006339, and known variants) (see SEQ ID NOS:1 and 2).

The term "HRH1" is used in reference to the gene, cDNA, mRNA or corresponding protein sequences of *homo sapiens* histamine receptor H1 (accession numbers NM_00861 and BC060802, and known variants of both of these) (see SEQ ID NOS:3, 4 and 5).

The term "NP" is used in reference to the gene, cDNA, mRNA or corresponding protein sequences of *homo sapiens* nucleoside phosphorylase (accession number NM_000270, and known variants) (see SEQ ID NOS:6 and 7).

The term "YES" or "c-YES" or"YES1" is used in reference to the gene, cDNA, mRNA or corresponding protein sequences of *homo sapiens* v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 (accession number NM_005433, and known variants) (see SEQ ID NOS:8 and 9).

The phrase "HIV-mediated cellular effects, conditions and diseases" or "HIV-related (or mediated) conditions or diseases" refers to those illnesses and conditions included in, but not necessarily limited to the CDC 1993 AIDS surveillance case definition, as follows: Bacillary angiomatosis; Candidiasis of bronchi, trachea, or lungs; Candidiasis, esophageal; Candidiasis, oropharyngeal (thrush); Candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy; Cervical dysplasia (moderate or severe)/cervical carcinoma in situ; Cervical cancer, invasive *; Coccidioidomycosis, disseminated or extrapulmonary; Constitutional symptoms, such as fever (38.5 C) or diarrhea lasting greater than 1 month; Cryptococcosis, extrapulmonary; Cryptosporidiosis, chronic intestinal (greater than 1 month's duration); Cytomegalovirus disease (other than liver, spleen, or nodes); Cytomegalovirus retinitis (with loss of vision); Encephalopathy, HIV-related; Herpes simplex: chronic ulcer(s) (greater than 1 month's duration); or bronchitis, pneumonitis, or esophagitis; Hairy leukoplakia, oral; Herpes zoster (shingles), involving at least two distinct episodes or more than one dermatome; Histoplasmosis,

disseminated or extrapulmonary; Idiopathic thrombocytopenic purpura; Isosporiasis, chronic intestinal (greater than 1 month's duration); Kaposi's sarcoma; Listeriosis; Lymphoma, Burkitt's (or equivalent term); Lymphoma, immunoblastic (or equivalent term); Lymphoma, primary, of brain; Mycobacterium avium complex or M. kansasii, disseminated or extrapulmonary; Mycobacterium tuberculosis, any site (pulmonary or extrapulmonary); Mycobacterium, other species or unidentified species, disseminated or extrapulmonary; Peripheral neuropathy; Pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess; Pneumocystis carinii pneumonia; Pneumonia, recurrent; Progressive multifocal leukoencephalopathy; Salmonella septicemia, recurrent; Toxoplasmosis of brain; and Wasting syndrome due to HIV. The underlying premise for the use of these phrases in the present inventive context is that the CD4+ T-lymphocyte is the primary target for HIV infection because of the affinity of the virus for the CD4 surface marker. The CD4+ T-lymphocyte coordinates a number of important immunologic functions, and a loss of these functions results in progressive impairment of the immune response. Studies of the natural history of HIV infection have documented a wide spectrum of disease manifestations, ranging from asymptomatic infection to life-threatening conditions characterized by severe immunodeficiency, serious opportunistic infections, and cancers. Other studies have shown a strong association between the development of life-threatening opportunistic illnesses and the absolute number (per microliter of blood) or percentage of CD4+ T- lymphocytes. As the number of CD4+ T-lymphocytes decreases, the risk and severity of opportunistic illnesses increase. Accordingly, treatment of HIV infection and/or replication address many related conditions and illnesses according to the present invention.

Myeloid cell line model system for in vivo HIV-related effects. Inventive HIV-related therapeutic targets were identified by the use of the monocyte/macrophage cell line "THP-1". This is a human myeloid cell line derived from an acute monocytic leukemia. The cells grow in suspension and exhibit many features of monocytes, including but not limited to the ability to differentiate into macrophage-like cells following phorbol ester treatment. They express CD4

and HIV co-receptors, and are susceptible to HIV infection. THP-1 were chosen for this study because they more closely represent native monocyte/macrophages than other available human myeloid cell lines (*e.g.*, HL60, U937, KG-1 or HEL cells) (*for review see* Auwerx, J. *Experimentia* 47:22-31, 1991, entitled "The human leukemia cell line, THP-1: a multi-facetted model for the study of monocyte-macrophage differentiation"). THP-1 cells are available through the ATCC.

Additionally, the T cell line "MT-2" was employed in the present model system. This is a human T cell line leukemia cell line. The cells grow in suspension and are very susceptible to acute infection with HIV. The cells can be efficiently loaded with antisense oligonucleotides. In addition, they have been shown by other investigators to provide a sensitive and reproducible system to test antiviral agents (*see, e.g.*, Haertle et al, *J. Biol. Chem.* 263:5870-5875, 1988). MT-2 cells are available through the NIH AIDS Research and Reference Reagent Program.

Finally, the HIV-1 strain used in the model system was the 89.6 strain. This is a dual tropic (X4/R5) HIV strain, meaning that it can infect cells utilizing CD4 and either the CXCR4 or the CCR5 co-receptor. Thus, both T cells (*e.g.*, MT-2) and macrophages (*e.g.*, THP-1) are susceptible to infection by the same virus strain. HIV-1 89.6 was originally provided by the investigator who isolated and characterized it, Dr Ronald Collman (Collman et al, *J. Virology* 66:7517, 1992). Applicant's expanded the virus by culture in PBMC, and concentrated it for use in the inventive system as described in EXAMPLE 2, herein below.

Identification of HIV-induced cellular genes using microarrays. Cellular genes involved in HIV replication cycle were identified by using DNA microarrays to examine the differential gene expression profiles of THP1 monocytes before and after HIV-infection. Such microarray technology is well known in the art (*see, e.g.*, Moses et al., *J. Virol.* 76:8383-8399, 2002; WO 02/10339 A2, published 07 February 2002; Salunga et al., *In* M. Schena (ed.), DNA microarrays, A practical approach; Oxford Press, Oxford, United Kingdom, 1999; *and see* Simmen et al., *Proc. Natl. Acad. Sci. USA* 98:7140-7145, 2001; all of which are incorporated by reference herein), and can be performed using commercially available arrays (*e.g.*, Affymetrix U133A,

U133B and U95A GeneChip® arrays) (Affymetrix, Santa Clara, CA). The Human Genome U133 (HG-U133) set, consists of two GeneChip® arrays, and contains almost 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes (Affymetrix technical information). The set design uses sequences selected from GenBank®, dbEST, and RefSeq (*Id*).

Specifically, as described in detail under EXAMPLE 2 herein, nucleic acid microarray technology was used for gene expression profiling of HIV-infected (synchronized) THP1 cells, relative to non-infected control cells, to identify cellular genes whose expression is regulated by HIV. Each of the TPH1 cell infected/uninfected sample comparisons resulted in multiple probe sets with increased expression, with most showing increased expression in duplicate infections. Approximately 20 genes with increased transcription in at least two adjacent time points across two infections were selected (EXAMPLE 2) for validation as described in EXAMPLE 3, herein below.

Validation of therapeutic targets by gene silencing using gene-specific PMO antisense compounds. Particular HIV-regulated or HIV-induced gene sequences were identified as *validated* therapeutic targets by specific gene silencing using PMO (phosphorodiamidate morpholino Oligomers) antisense oligonucleotide inhibition in combination with measuring the effects of such gene silencing using MT-2-, or THP1-HIV replication assays (EXAMPLE 3, below). Silencing of particular target genes precluded progression of HIV replication, as measured by decreased production of HIV-1 gag protein p24, thus validating such genes as therapeutic intervention targets.

Therapeutic utility. According to the present invention, PMO-mediated gene silencing using the THP1 and MT-2 system with HIV 89.6 (or HIV MN) not only provides validation of therapeutically-significant targets, but also provides gene-specific modulators of HIV-induced cellular gene expression that have therapeutic utility. PMOs (*see, e.g.*, Summerton, et al., *Antisense Nucleic Acid Drug Dev.* 7:63-70, 1997; and Summerton & Weller, *Antisense Nucleic Acid Drug Dev.* 7:187-95, 1997) represent a class of art-recognized antisense drugs for treating

various diseases, including cancer. For example, Arora et al. (*J. Pharmaceutical Sciences* 91:1009-1018, 2002) demonstrated that oral administration of *c-myc*-specific and CYP3A2-specific PMOs inhibited *c-myc* and CYP3A2 gene expression, respectively, in rat liver by an antisense mechanism of action. Likewise, Devi G.R. (*Current Opinion in Molecular Therapeutics* 4:138-148, 2002) discusses treatment of prostate cancer with various PMO therapeutic agents).

Likewise, siRNA" or "RNAi" agents are emerging as a new class of art-recognized drugs (*see e.g.*: U.S. Patent 6,506,559; Milhavet et al., *Pharmacological Reviews* 55:629-648, 2003; and Gitlin et al., *J. Virol.* 77:7159-7165, 2003; incorporated herein by reference).

Accordingly, the present invention provides therapeutic compositions, and methods for modulation of HIV infection and/or replication, comprising inhibition of HIV-induced celllar gene expression (*e.g.*, inhibition of the expression of validated HIV-induced genes, reducing or preventing the expression of mRNA from validated HIV-induced genes, or reducing the biological activity of corresponding HIV-induced cellular gene products).

Additional embodiments provide screening assays for compounds useful to modulate HIV infection and/or replication.

Further embodiments provide diagnostic or prognostic assays for HIV infection and/or replication.

<u>Preferred Inventive Modulators, Compositions, Utilities and Expression Vectors</u>

Modulators of HIV-induced gene expression. Particular embodiments provide modulators of HIV-induced cellular gene expression. Preferably, inventive modulators are directed to one or more validated HIV-induced cellular gene targets, the expression of which is required, at least to some extent, for HIV-mediated effects on cellular proliferation and phenotype.

Inventive modulators include, but are not limited to, antisense molecules, siRNA, ribozymes, antibodies or antibody fragments, proteins or polypeptides as well as small molecules. Particular HIV-induced gene expression modulators, such as gene-specific antisense,

siRNA, and ribozyme molecules, and antibodies and epitope-binding fragments thereof, are *inhibitors* of HIV-induced gene expression, or of the biological activity of proteins encoded thereby.

Preferably, inventive antisense molecules are oligonucleotides of about 10 to 35 nucleotides in length that are targeted to a nucleic acid molecule corresponding to a HIV-induced gene sequence, wherein the antisense molecule inhibits the expression of at least one HIV-induced gene sequence. Antisense compounds useful to practice the invention include oligonucleotides containing art-recognized modifed backbones or non-natural internucleoside linkages, modified sugar moieties, or modified nucleobases.

Preferred antisense molecules or the complements thereof comprise at least 10, at least 15, at least 17, at least 20 or at least 25, and preferably less than about 35 consecutive complementary nucleotides of, or hybridize under stringent or highly stringent conditions to at least one of the nucleic acid sequences from the group consisting of: SEQ ID NO:1 (cDNA/mRNA for HMG20B; *homo sapiens* high-mobility group 20B; accession number NM_006339, and known variants); SEQ ID NO:3 and SEQ ID NO:5 (cDNA/mRNA for HRH1; *homo sapiens* histamine receptor H1; accession numbers NM_00861 and BC060802, and known variants of both of these); SEQ ID NO:6 (cDNA/mRNA for NP; *homo sapiens* nucleoside phosphorylase; accession number NM_000270, and known variants); and SEQ ID NO:8 (cDNA/mRNA for YES1; *homo sapiens* v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1; accession number NM_005433, and known variants); or to the complements thereof. Preferably, such antisense molecules are PMO (phosphorodiamidate morpholino Oligomers*)* antisense molecules.

Thus, the present invention includes nucleic acids that hybridize under stringent hybridization conditions, as defined below, to all or a portion of the validated HIV-induced cellular gene sequences represented by the cDNA sequences of SEQ ID NOS:1, 3, 5, 6 and 8, or the complements thereof. The hybridizing portion of the hybridizing nucleic acids is typically at least 10, 15, 17, 20, 25, 30 or 35 nucleotides in length. Preferably, the hybridizing portion of the hybridizing nucleic acid is at least 80%, at least 95%, or at least 98% identical to the sequence of a portion or all of the cDNA sequences of SEQ ID NOs:1, 3, 5, 6 and 8, or to the complements thereof.

Hybridizing nucleic acids of the type described herein can be used, for example, as an inventive therapeutic modulator of HIV-induced gene expression, a cloning probe, a primer (*e.g.*, a PCR primer), or a diagnostic and/or prognostic probe or primer. Preferably, hybridization of the oligonucleotide probe to a nucleic acid sample is performed under stringent conditions. Nucleic acid duplex or hybrid stability is expressed as the melting temperature or Tm, which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions.

For sequences that are related and substantially identical to the probe, rather than identical, it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (*e.g.*, SSC or SSPE). Then, assuming that 1% mismatching results in a 1°C decrease in the Tm, the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having > 95% identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in Tm can be between 0.5°C and 1.5°C per 1% mismatch.

Stringent conditions, as defined herein, involve hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature, or involve the art-recognized equivalent thereof. Moderately stringent conditions, as defined herein, involve including washing in 3x SSC at 42°C, or the art-recognized equivalent thereof. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Guidance regarding such conditions is available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

Antisense molecules preferably comprise at least 17 or at least 20, or at least 25, and preferably less than about 35 consecutive complementary nucleotides of, or hybridize under stringent conditions to at least one of the nucleic acid sequences from the group consisting of SEQ ID NOS:1, 3, 5, 6 and 8, and complements thereof. Preferably, such antisense molecules are PMO antisense molecules. Preferred representative antisense molecules are provided herein as:

SEQ ID NO:10	(HMG20)	5'-cgcccagcatcttggtgatctcggg-3';
SEQ ID NO:11	(HRH1)	5'-gcgaaagagcagccgccagttatgg-3';

SEQ ID NO:12 (NP) 5'-cttcataggtgtatccgttctccat-3';

SEQ ID NO:13 (c-YES) 5'-tttctttacttttaatgcagcccat-3'; and

SEQ ID NO:14 (ARF1) 5'-atgcttgtggacaggtggaaggaca-3'.

Preferably, these antisense molecules are PMO antisense molecules.

Even more preferably, representative antisense molecules are provided herein as SEQ ID NOS:10, 11, 12, and 13, and these antisense molecules are preferably PMO antisense molecules.

The invention further provides a ribozyme capable of specifically cleaving at least one RNA specific to HMG20, HRH1, NP, and c-YES, and a pharmaceutical composition comprising the ribozyme.

The invention also provides small molecule modulators of HIV-induced gene expression, wherein particular modulators are inhibitors capable of reducing the expression of at least one HIV-induced gene, reducing or preventing the expression of mRNA from at least one HIV-induced gene, or reducing the biological activity of at least one HIV-induced gene product. Preferably, the HIV-induced gene is selected from the group consisting of HMG20, HRH1, NP, and c-YES.

Compositions. Further embodiments provide compositions that comprise one or more modulators of HIV-induced gene expression (or modulators of biological activity of HIV-induced gene products) in a pharmaceutically acceptable carrier or diluent.

Particular embodiments provide a pharmaceutical composition for inhibiting HIV-induced gene expression, comprising an antisense oligonucleotide according to the invention in a mixture with a pharmaceutically acceptable carrier or diluent.

Further provided is a composition comprising a therapeutically effective amount of an inhibitor of a HIV-induced gene product (*e.g.*, protein) in a pharmaceutically acceptable carrier. In certain embodiments, the composition comprises two or more HIV-induced gene product inhibitors. Preferably, the HIV-induced gene product is selected from: the nucleic acid group consisting of SEQ ID NOS:1, 3, 5, 6 and 8, and combinations thereof, corresponding to HMG20, HRH1, NP, and c-YES, and combinations thereof, respectively; or from the protein group consisting of SEQ ID NOS:2, 4, 7 and 9, and combinations thereof, corresponding to HMG20, HRH1, NP, and c-YES, respectively.

In particular composition embodiments, the HIV-induced gene inhibitor is an antisense molecule, and in specific embodiments the antisense molecule or the complement thereof

comprises at least 10, 15, 17, 20 or 25 consecutive nucleic acids of, or hybridizes under stringent conditions to at least one of the nucleic acid sequences from the group consisting of SEQ ID NOS:1, 3, 5, 6, and 8, and complements thereof. Preferably, such antisense molecules are PMO antisense molecules. Preferably, the antisense molecules comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOS:10-13.

Methods and uses. Particular embodiments of the present invention provide methods of modulating HIV-induced gene expression or biological activity of HIV-induced gene products in HIV-infected cells.

The invention provides a method of inhibiting the expression of HIV-induced cellular genes in human cells or tissues comprising contacting the cells or tissues *in vivo* (also *ex vivo*, or *in vitro*) with an antisense compound or a ribozyme of 10 to 35 nucleotides in length targeted to a nucleic acid molecule encoding a HIV-induced gene product so that expression of the human HIV-induced gene product is inhibited. Preferably, the HIV-induced gene is selected from the group consisting of: HMG20B (*homo sapiens* high-mobility group 20B, accession number NM_006339, and known variants); HRH1 (*homo sapiens* histamine receptor H1, accession numbers NM_00861 and BC060802, and known variants of both of these); NP (*homo sapiens* nucleoside phosphorylase, accession number NM_000270, and known variants); and YES1(*homo sapiens* v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1, accession number NM_005433, and known variants); or combinations thereof. Preferably, the antisense compounds are PMOs.

The invention additionally provides a method of modulating HIV replication in cells comprising contacting the cells *in vivo* (also *ex vivo*, or *in vitro*) with an inventive antisense compound or ribozyme of 10 to 35 nucleotides in length targeted to a nucleic acid molecule encoding a HIV-induced gene product so that expression of the human HIV-induced gene product is inhibited and HIV replication is inhibited.

The invention provides for the use of a modulator of HIV-induced gene expression according to the invention to prepare a medicament for modulating HIV replication, HIV-mediated cell proliferation and/or HIV-mediated cellular phenotype.

Additional embodiments provide a method of inhibiting HIV-induced gene expression or encoded biological activity in a mammalian cell, comprising administering to the cell an inhibitor of HIV-induced gene expression (or of encoded biological activity), and in a specific

embodiment of the method, the inhibitor is a target gene-specific antisense molecule. Preferably, the antisense molecule is a PMO antisense molecule. Preferably, the antisense molecules comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOS:10-13.

The invention also provides a method of inhibiting HIV-induced gene expression in a subject, comprising administering to said subject, in a pharmaceutically effective vehicle, an amount of an antisense oligonucleotide which is effective to specifically hybridize to all or part of a selected target nucleic acid sequence derived from said HIV-induced gene. In preferred embodiments of this method, the target-specific antisense oligonucleotide is selected from the group consisting of SEQ ID NOS:10, 11, 12 and 13. Preferably the antisense oligonucleotides are PMO antisense compounds.

The invention further provides a method of treating HIV-related conditions or disease, comprising administering to a mammalian cell a modulator of HIV-induced gene expression such that the neoplastic condition or disease is reduced in severity.

As discussed herein below in EXAMPLES 1-4 (particularly, EXAMPLE 4) additional embodiments provide screening assays for identification of compounds useful to modulate HIV infection, comprising: contacting HIV-infected cells with a test agent; measuring, using a suitable assay, expression of at least one validated HIV-induced cellular gene sequence; and determining whether the test agent inhibits said validated gene expression relative to control cells not contacted with the test agent, whereby agents that inhibit said validated gene expression are identified as compounds useful to modulate HIV infection.

Preferably, expression of at least one validated HIV-induced cellular gene sequence is expression of respective mRNA, or expression of the protein encoded thereby.

Preferably, the at least one validated HIV-induced cellular gene sequence is selected from the cDNA and protein sequence group consisting of HMG20, HRH1, NP, and c-YES, and combinations thereof (*i.e.*, consisting of SEQ ID NOS:1-9, and combinations thereof).

Preferably, agents that inhibit said validated gene expression are further tested for the ability to modulate HIV infection and/or replication, HIV-mediated cellular proliferation and/or HIV-mediated cellular phenotype, conditions or diseases.

Further embodiments provide diagnostic or prognostic assays for HIV infection and/or replication comprising: obtaining a cell sample from a subject suspected of having HIV; measuring expression of at least one validated HIV-inducible cellular gene sequence; and

determining whether expression of the at least one validated gene is induced relative to non-HIV-infected control cells, whereby a diagnosis is, at least in part, afforded.

Preferably, the at least one validated HIV-inducible cellular gene is selected from the cDNA and protein sequence group consisting of HMG20, HRH1, NP, and c-YES, and combinations thereof (*i.e.*, consisting of SEQ ID NOS:1-9, and combinations thereof).

Preferably, measuring said expression is of two or more validated HIV-inducible cellular gene sequences. Preferably, measurement of said expression is by use of high-throughput microarray methods.

Polynucleotides and expression vectors. Particular embodiments provide an isolated polynucleotide with a sequence comprising a transcriptional initiation region and a sequence encoding a HIV-induced gene-specific antisense oligonucleotide at least 10, 15, 17, 20 or 25 nucleotides in length, and a recombinant vector comprising this polynucleotide (*e.g.*, expression vector). Preferably, the antisense oligonucleotide of said polynucleotide comprises a sequence selected from the group consisting of SEQ ID NOS:10-13. Preferably, the transcriptional initiation region is a strong constitutively expressed mammalian pol III-or pol II-specific promoter, or a viral promoter.

Additional and Preferred Oligonucleotide Modulators

Included within the scope of the invention are oligonucleotides capable of hybridizing with HIV-induced gene DNA or RNA, referred to herein as the 'target' polynucleotide. An oligonucleotide need not be 100% complementary to the target polynucleotide, as long as specific hybridization is achieved. The degree of hybridization to be achieved is that which interferes with the normal function of the target polynucleotide, be it transcription, translation, pairing with a complementary sequence, or binding with another biological component such as a protein. An antisense oligonucleotide, including a preferred PMO antisense oligonucleotide, can interfere with DNA replication and transcription, and it can interfere with RNA translocation, translation, splicing, and catalytic activity.

The invention includes within its scope any oligonucleotide of about 10 to about 35 nucleotides in length, including variations as described herein, wherein the oligonucleotide hybridizes to a HIV-induced target sequence, including DNA or mRNA, such that an effect on the normal function of the polynucleotide is achieved. The oligonucleotide can be, for example,

10, 15, 17, 20, 22, 23, 25, 30 or 35 nucleotides in length. Oligonucleotides larger than 35 nucleotides are also contemplated within the scope of the present invention, and may for example, correspond in length to a complete target cDNA (*i.e.*, mRNA) sequence, or to a significant or substantial portion thereof.

Antisense oligonucleotides. As described above, preferred antisense molecules are represented by SEQ ID NOS:10-13, and combinations thereof.

Examples of representative preferred antisense compounds useful in the invention are based on SEQ ID NOS:1, 3, 5, 6, 8 and 10-13, and include oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those retaining a phosphorus atom in the backbone, and those that do not have a phosphorus atom in the backbone.

Preferred modified oligonucleotide backbones include phosphorothioates or phosphorodithioate, chiral phosphorothioates, phosphotriesters and alkyl phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including methylphosphonates, 3′-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoroamidates or phosphordiamidates, including 3′-amino phosphoroamidate and aminoalkylphosphoroamidates, and phosphorodiamidate morpholino oligomers (PMOs), thiophosphoroamidates, phosphoramidothioates, thioalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3′-5′ linkages, 2′-5′ linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3′-5′ to 5′-3′ or 2′-5′ to 5′-2′. Various salts, mixed salts and free acid forms are also included.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to arabinose, 2-fluoroarabinose, xylulose, hexose and 2'-O-methyl sugar moieties.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-

methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine (*see also* U.S. 5,958,773 and patents disclosed therein).

Examples of inventive antisense oligonucleotides of length X (in nucleotides), as indicated by polynucleotide positions with reference to, *e.g.*, SEQ ID NO:1, include those corresponding to sets of consecutively overlapping oligonucleotides of length X, where the oligonucleotides within each consecutively overlapping set (corresponding to a given X value) are defined as the finite set of Z oligonucleotides from nucleotide positions:

n to (n + (X-1));

where n=1, 2, 3,...(Y-(X-1));

where Y equals the length (nucleotides or base pairs) of SEQ ID NO:1 (1,232);

where X equals the common length (in nucleotides) of each oligonucleotide in the set (*e.g.*, X=20 for a set of consecutively overlapping 20-mers); and

where the number (Z) of consecutively overlapping oligomers of length X for a given SEQ ID NO of length Y is equal to Y-(X-1). For example Z=1,232-19=1,213 for SEQ ID NO:1, where X=20.

Examples of inventive 20-mer oligonucleotides include the following set of 1,213 oligomers, indicated by polynucleotide positions with reference to SEQ ID NO:1 (HMG20B cDNA):

1-20, 2-21, 3-22, 4-23, 5-24, ...1211-1230, 1212-1231 and 1213-1232.

Likewise, examples of 25-mer oligonucleotides include the following set of 1,208 oligomers, indicated by polynucleotide positions with reference to SEQ ID NO:1:

1-25, 2-26, 3-27, 4-28, 5-29, ...1206-1230, 1207-1231 and 1208-1232.

The present invention encompasses, for *each* validated target sequence (*e.g.*, for SEQ ID NOS:1, 3, 5, 6, and 8, and the complements thereof), multiple consecutively overlapping sets of oligonucleotides or modified oligonucleotides of length X, where, *e.g.*, X= 10, 17, 20, 22, 23, 25, 30 or 35 nucleotides.

Preferred sets of such oligonucleotides or modified oligonucleotides of length X are those consecutively overlapping sets of oligomers corresponding to SEQ ID NOS:1, 3, 5, 6, and 8, and to the complements thereof. Included in these preferred sets are the preferred oligomers corresponding to SEQ ID NOS:10-13.

The antisense oligonucleotides of the invention can also be modified by chemically linking the oligonucleotide to one or more moieties or conjugates to enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide. Such moieties or conjugates include lipids such as cholesterol, cholic acid, thioether, aliphatic chains, phospholipids, polyamines, polyethylene glycol (PEG), palmityl moieties, and others as disclosed in, for example, United States Patent Numbers 5,514,758, 5,565,552, 5,567,810, 5,574,142, 5,585,481, 5,587,371, 5,597,696 and 5,958,773. Thus, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating or modulating transport across the cell membrane (Letsinger et al., *Proc. Natl. Acad. Sci. USA* 86:6553-6556, 1989; Lemaitre et al., *Proc. Natl. Acad. Sci. USA* 84:648-652, 1987; PCT WO88/09810, published Dec. 15, 1988) or the blood-brain barrier (PCT WO89/10134, published Apr. 25, 1988), or the nuclear membrane, and may include hybridization-triggered cleavage agents (Krol et al., *BioTechniques* 6:958-976, 1988) or intercalating agents (Zon, *Pharm. Res.* 5:539-549, 1988). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization-triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Chimeric antisense oligonucleotides are also within the scope of the invention, and can be prepared from the present inventive oligonucleotides using the methods described in, for example, United States Patent Numbers 5,013,830, 5,149,797, 5,403,711, 5,491,133, 5,565,350, 5,652,355, 5,700,922 and 5,958,773.

Preferred antisense oligonucleotides in addition to those of SEQ ID NOS:10-14 are selected by routine experimentation using, for example, assays described in the present Examples. Although the inventors are not bound by a particular mechanism of action, it is

believed that the antisense oligonucleotides achieve an inhibitory effect by binding to a complementary region of the target polynucleotide within the cell using Watson-Crick base pairing. Where the target polynucleotide is RNA, experimental evidence indicates that the RNA component of the hybrid is cleaved by RNase H (Giles, R.V. et al., *Nuc. Acids Res.* (1995) *23*:954-961; U.S. Patent No. 6,001,653). Generally, a hybrid containing 10 base pairs is of sufficient length to serve as a substrate for RNase H. However, to achieve specificity of binding, it is preferable to use an antisense molecule of at least 17 nucleotides, as a sequence of this length is likely to be unique among human genes.

Antisense approaches comprise the design of oligonucleotides (either DNA or RNA) that are complementary to the target gene sequence (*e.g.*, mRNA). The antisense oligonucleotides bind to the complementary mRNA transcripts and prevent translation. Absolute complementarily, although preferred, is not required. A sequence "complementary" to a portion or region of the target mRNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize depends on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA are accommodated without compromising stable duplex (or triplex, as the case may be) formation. One skilled in the art ascertains a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

As disclosed in U.S. Patent No. 5,998,383, incorporated herein by reference, the oligonucleotide is selected such that the sequence exhibits suitable energy related characteristics important for oligonucleotide duplex formation with their complementary targets, and shows a low potential for self-dimerization or self-complementation (Anazodo et al., *Biochem. Biophys. Res. Commun.* (1996) *229*:305-309). The computer program OLIGO (Primer Analysis Software, Version 3.4), is used to determined antisense sequence melting temperature, free energy properties, and to estimate potential self-dimer formation and self-complementarity properties. The program allows the determination of a qualitative estimation of these two parameters (potential self-dimer formation and self-complementary) and provides an indication of "no potential" or "some potential" or "essentially complete potential." Preferably, segments of

validated HIV-induced gene sequences are selected that have estimates of no potential in these parameters. However, segments that have "some potential" in one of the categories nonetheless can have utility, and a balance of the parameters is routinely used in the selection.

While antisense nucleotides complementary to the coding region sequence of a mRNA are used in accordance with the invention, those complementary to the transcribed, untranslated region, or translational initiation site region are sometimes preferred. Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5'-untranslated sequence (up to and including the AUG initiation codon), frequently work most efficiently at inhibiting translation. However, sequences complementary to the 3'-untranslated sequences, or other regions of mRNAs are also effective at inhibiting translation of mRNAs (*see e.g.*, Wagner, *Nature* 372:333-335, 1994). In the antisense art a certain degree of routine experimentation is required to select optimal antisense molecules for particular targets. To be effective, the antisense molecule preferably is targeted to an accessible, or exposed, portion of the target RNA molecule. Although in some cases information is available about the structure of target mRNA molecules, the current approach to inhibition using antisense is via experimentation.

Such experimentation can be performed routinely by transfecting or loading cells with an antisense oligonucleotide, followed by measurement of messenger RNA (mRNA) levels in the treated and control cells by reverse transcription of the mRNA and assaying of respective cDNA levels. Measuring the specificity of antisense activity by assaying and analyzing cDNA levels is an art-recognized method of validating antisense results. Routinely, RNA from treated and control cells is reverse-transcribed and the resulting cDNA populations are analyzed (Branch, A. D., *T.I.B.S.*(1998) *23*:45-50).

According to the present invention, antisense efficacy can be alternately determined by measuring the biological effects on cell growth, phenotype or viability as is known in the art, and as shown in the present Examples. According to the present invention, cultures of HIV-infected TPH1 cells or MT-2 cells were loaded with inventive oligonucleotides designed to target HIV-induced gene sequences. Preferred representative antisense oligonucleotides correspond to SEQ ID NOS:10-14. The effects of such loading on HIV replication were measured. Specifically, SEQ ID NOS:10-13 caused dramatic decreases in HIV replication, as measured by decreases in HIV gag 24 protein, a hallmark of *in vivo* HIV-related replication.

Ribozymes. Modulators of HIV-induced gene expression may be ribozymes. A ribozyme is an RNA molecule that specifically cleaves RNA substrates, such as mRNA, resulting in specific inhibition or interference with cellular gene expression. As used herein, the term ribozymes includes RNA molecules that contain antisense sequences for specific recognition, and an RNA-cleaving enzymatic activity. The catalytic strand cleaves a specific site in a target RNA at greater than stoichiometric concentration. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target mRNA (*i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts).

A wide variety of ribozymes may be utilized within the context of the present invention, including for example, the hammerhead ribozyme (for example, as described by Forster and Symons, *Cell* (1987) *48*:211-220; Haseloff and Gerlach, *Nature* (1988) *328*:596-600; Walbot and Bruening, *Nature* (1988) *334*:196; Haseloff and Gerlach, *Nature* (1988) *334*:585); the hairpin ribozyme (for example, as described by Haseloff et al., U.S. Patent No. 5,254,678, issued October 19, 1993 and Hempel et al., European Patent Publication No. 0 360 257, published March 26, 1990); and Tetrahymena ribosomal RNA-based ribozymes (*see* Cech et al., U.S. Patent No. 4,987,071). The Cech-type ribozymes have an eight-base pair active site that hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. Ribozymes of the present invention typically consist of RNA, but may also be composed of DNA, nucleic acid analogs (*e.g.*, phosphorothioates), or chimerics thereof (*e.g.*, DNA/RNA/RNA).

Ribozymes can be targeted to any RNA transcript and can catalytically cleave such transcripts (*see, e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246 to Cech et al.). According to certain embodiments of the invention, any such HIV-induced gene sequence-specific ribozyme, or a nucleic acid encoding such a ribozyme, may be delivered to a host cell to effect inhibition of HIV-induced gene expression. Ribozymes and the like may therefore be delivered to the host cells by DNA encoding the ribozyme linked to a eukaryotic promoter (*e.g.*, a strong constitutively expressed pol III- or pol II-specific promoter), or a eukaryotic viral promoter, such that upon introduction into the nucleus, the ribozyme will be directly transcribed.

Triple-helix formation. Alternatively, validated HIV-induced gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (*e.g.*, respective promoter and/or enhancers) to form triple helical structures that prevent transcription of the target gene (*see, e.g.*, Helen, *Anticancer Drug Des.*, 6:569-84, 1991; Helene et al., *Ann, N.Y. Acad. Sci.*, 660:27-36, 1992; and Maher, *Bioassays* 14:807-15, 1992).

siRNA. The invention, in particular aspects, contemplates introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. According to the present invention, inhibition is specific to the particular validated HIV-induced cellular gene expression product in that a nucleotide sequence from a portion of the validated sequence is chosen to produce inhibitory RNA. This process is effective in producing inhibition (partial or complete), and is validated gene-specific. In particular embodiments, the target cell containing the validate gene may be a human cell subject to infection by HIV (or cell-lines derived therefrom). Methods of preparing and using siRNA are generally disclosed in U.S. Patent 6,506,559, incorporated herein by reference (*see also* reviews by Milhavet et al., *Pharmacological Reviews* 55:629-648, 2003; and Gitlin et al., *J. Virol.* 77:7159-7165, 2003; incorporated herein by reference).

The siRNA may comprise one or more strands of polymerized ribonucleotide, and may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside

or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. Nucleic acid containing a nucleotide sequence identical to a portion of the validated gene sequence is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

For siRNA (RNAi), the RNA may be directly introduced into the cell (*i.e.*, intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express a RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a validated gene target. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern

hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, fluorescence activated cell analysis (FACS), and HIV viral infection and/or replication as described herein. For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Many such reporter genes are known in the art.

The phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

RNA containing a nucleotide sequences identical to a portion of a particular validated gene sequence are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence may be effective for inhibition. Sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (*e.g.*, University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of particular validated gene sequence is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the particular validated gene transcript (*e.g.*, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C. or 70°C. hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 20, 25, 50, 100, 200, 300 or 400 bases.

A 100% sequence identity between the RNA and a particular validated gene sequence is not required to practice the present invention. Thus the methods have the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

Particular validated gene sequence siRNA may be synthesized by art-recognized methods either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription in vivo or *in vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (*e.g.*, infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus.

RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3, T7, SP6). The use and production of an expression construct are known in the art (*e.g.*, WO 97/32016; U.S. Pat. Nos: 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

siRNA may be directly introduced into the cell (*i.e.*, intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced

orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The siRNA may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Suitable injection mixes are constructed so animals receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections are compared with equal masses of RNA (*i.e.*, dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible.

Proteins and Polypeptides

In addition to the antisense molecules, siRNA and ribozymes disclosed herein, inventive modulators of HIV-induced gene expression also include proteins or polypeptides that are effective in either reducing validated HIV-induced cellular gene expression or in decreasing one or more of the respective biological activities encoded thereby. A variety of art-recognized methods are used by the skilled artisan, through routine experimentation, to rapidly identify such modulators of HIV-induced gene expression. The present invention is not limited by the following exemplary methodologies.

Inhibitors of HIV-induced biological activities encompass those proteins and/or polypeptides that interfere with said biological activities. Such interference may occur through direct interaction with active domains of the proteins of validated gene targets, or indirectly through non- or un-competitive inhibition such as via binding to an allosteric site. Accordingly, available methods for identifying proteins and/or polypeptides that bind to proteins of validated gene targets may be employed to identify lead compounds that may, through the methodology disclosed herein, be characterized for their inhibitory activity.

Methods for detecting and analyzing protein-protein interactions are described in the art, and are thus available to skilled artisans (*reviewed in* Phizicky, E.M. et al., *Microbiological Reviews* (1995) *59*:94-123 incorporated herein by reference. Such methods include, but are not limited to physical methods such as, *e.g.*, protein affinity chromatography, affinity blotting,

immunoprecipitation and cross-linking as well as library-based methods such as, *e.g.*, protein probing, phage display and two-hybrid screening. Other methods that may be employed to identify protein-protein interactions include genetic methods such as use of extragenic suppressors, synthetic lethal effects and unlinked noncomplementation. Exemplary methods are described in further detail below.

Inventive inhibitors of proteins of validated gene targets (validated proteins) may be identified through biological screening assays that rely on the direct interaction between the a validated protein (*e.g.*, SEQ ID NOS:2, 4, 7, and 9) and a panel or library of potential inhibitor proteins. Biological screening methodologies, including the various "n-hybrid technologies," are described in, for example, Vidal, M. et al., *Nucl. Acids Res.* (1999) *27*(4):919-929; Frederickson, R.M., *Curr. Opin. Biotechnol.* (1998) *9*(1):90-6; Brachmann, R.K. et al., *Curr. Opin. Biotechnol.* (1997) *8*(5):561-568; and White, M.A., *Proc. Natl. Acad. Sci. U.S.A.* (1996) *93*:10001-10003 each of which is incorporated herein by reference.

The two-hybrid screening methodology may be employed to search new or existing target cDNA libraries for inhibitory proteins. The two-hybrid system is a genetic method that detects protein-protein interactions by virtue of increases in transcription of reporter genes. The system relies on the fact that site-specific transcriptional activators have a DNA-binding domain and a transcriptional activation domain. The DNA-binding domain targets the activation domain to the specific genes to be expressed. Because of the modular nature of transcriptional activators, the DNA-binding domain may be severed from the otherwise covalently linked transcriptional activation domain without loss of activity of either domain. Furthermore, these two domains may be brought into juxtaposition by protein-protein contacts between two proteins unrelated to the transcriptional machinery. Thus, two hybrids are constructed to create a functional system. The first hybrid, *i.e.*, the bait, consists of a transcriptional activator DNA-binding domain fused to a protein of interest (*e.g.*, SEQ ID NOS:2, 4, 7, and 9, or fragments thereof). The second hybrid, the target, is created by the fusion of a transcriptional activation domain with a library of proteins or polypeptides. Interaction between the bait protein and a member of the target library

results in the juxtaposition of the DNA-binding domain and the transcriptional activation domain and the consequent up-regulation of reporter gene expression.

A variety of two-hybrid based systems are available to the skilled artisan that most commonly employ either the yeast Gal4 or *E. coli* LexA DNA-binding domain (BD) and the yeast Gal4 or herpes simplex virus VP16 transcriptional activation domain. Chien, C.-T. et al., *Proc. Natl. Acad. Sci. U.S.A.* (1991) *88*:9578-9582; Dalton, S. et al., *Cell* (1992) *68*:597-612; Durfee, T.K. et al., *Genes Dev.* (1993) *7*:555-569; Vojtek, A.B. et al., *Cell* (1993) *74*:205-214; and Zervos, A.S. et al., *Cell* (1993) *72*:223-232. Commonly used reporter genes include the *E. coli lacZ* gene as well as selectable yeast genes such as *HIS3* and *LEU2*. Fields, S. et al., *Nature (London)* (1989) *340*:245-246; Durfee, T.K., *supra*; and Zervos, A.S., *supra*. A wide variety of activation domain libraries is readily available in the art such that the screening for interacting proteins may be performed through routine experimentation.

Suitable bait proteins for the identification of inhibitors of validated proteins are designed based on the validated sequences presented herein as SEQ ID NO:2, 4, 7 and 9. Such bait proteins include either the full-length validated protein, or fragments thereof.

Plasmid vectors, such as, *e.g.*, pBTM116 and pAS2-1, for preparing validated protein bait constructs and target libraries are readily available to the artisan and may be obtained from such commercial sources as, *e.g.*, Clontech (Palo Alto, CA), Invitrogen (Carlsbad, CA) and Stratagene (La Jolla, CA). These plasmid vectors permit the in-frame fusion of cDNAs with the DNA-binding domains as LexA or Gal4BD, respectively.

Validated protein inhibitors of the present invention may alternatively be identified through one of the physical or biochemical methods available in the art for detecting protein-protein interactions.

For example, affinity chromatography may be used to identify potential inhibitors of validated proteins, by virtue of specific retention of such potential inhibitors to validated proteins, or to fragments thereof covalently or non-covalently coupled to a solid matrix such as, *e.g.*, Sepharose beads. The preparation of protein affinity columns is described in, for example,

Beeckmans, S. et al., *Eur. J. Biochem.* (1981) *117*:527-535 and Formosa, T. et al., *Methods Enzymol.* (1991) *208*:24-45. Cell lysates containing the full complement of cellular proteins may be passed through a validated protein affinity column. Proteins having a high affinity for the validated protein will be specifically retained under low-salt conditions while the majority of cellular proteins will pass through the column. Such high affinity proteins may be eluted from the immobilized validated protein, or fragment thereof under conditions of high-salt, with chaotropic solvents or with sodium dodecyl sulfate (SDS). In some embodiments, it may be preferred to radiolabel the cells prior to preparing the lysate as an aid in identifying the validated protein-specific binding proteins. Methods for radiolabeling mammalian cells are well known in the art and are provided, *e.g.*, in Sopta, M. et al., *J. Biol. Chem.* (1985) *260*:10353-10360.

Suitable validated proteins for affinity chromatography may be fused to a protein or polypeptide to permit rapid purification on an appropriate affinity resin. For example, a validated protein cDNA may be fused to the coding region for glutathione S-transferase (GST) which facilitates the adsorption of fusion proteins to glutathione-agarose columns. Smith et al., *Gene* (1988) *67*:31-40. Alternatively, fusion proteins may include protein A, which can be purified on columns bearing immunoglobulin G; oligohistidine-containing peptides, which can be purified on columns bearing Ni^{2+}; the maltose-binding protein, which can be purified on resins containing amylose; and dihydrofolate reductase, which can be purified on methotrexate columns. One such tag suitable for the preparation of validate protein fusion proteins is the epitope for the influenza virus hemagglutinin (HA) against which monoclonal antibodies are readily available and from which antibodies an affinity column may be prepared.

Proteins that are specifically retained on a validated protein affinity column may be identified after subjecting to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Thus, where cells are radiolabeled prior to the preparation of cell lysates and passage through the validated protein affinity column, proteins having high affinity for the particular validate protein may be detected by autoradiography. The identity of particular validated protein-specific binding proteins may be determined by protein sequencing techniques that are readily available to the

skilled artisan, such as those described by Mathews, C.K. et al., *Biochemistry*, The Benjamin/Cummings Publishing Company, Inc. pp. 166-170 (1990).

Antibodies or Antibody Fragments

Inhibitors of HIV-induced gene expression of the present invention include antibodies and/or antibody fragments that are effective in reducing HIV-induced gene expression and/or reducing the biological activity encoded thereby. Suitable antibodies may be monoclonal, polyclonal or humanized monoclonal antibodies. Antibodies may be derived by conventional hybridoma based methodology, from antisera isolated from validated protein inoculated animals or through recombinant DNA technology. Alternatively, inventive antibodies or antibody fragments may be identified *in vitro* by use of one or more of the readily available phage display libraries. Exemplary methods are disclosed herein.

In one embodiment of the present invention, validated protein inhibitors are monoclonal antibodies that may be produced as follows. Validated proteins (*e.g.*, SEQ ID NOS:2, 4, 7 and 9) may be produced, for example, by expression of the respective cDNAs (*e.g.*, SEQ ID NOS:1, 3, 5, 6, and 8, respectively) in a baculovirus based system. By this method, validated protein cDNAs (SEQ ID NOS:1, 3, 5, 6, and 8) or epitope-bearing fragments thereof are ligated into a suitable plasmid vector that is subsequently used to transfect Sf9 cells to facilitate protein production. In addition, it may be advantageous to incorporate an epitope tag or other moiety to facilitate affinity purification of the validated protein. Clones of Sf9 cells expressing a particular validated protein are identified, *e.g.*, by enzyme-linked immunosorbant assay (ELISA), lysates are prepared and the validated protein purified by affinity chromatography. The purified validated protein is, for example, injected intraperitoneally, into BALB/c mice to induce antibody production. It may be advantageous to add an adjuvant, such as Freund's adjuvant, to increase the resulting immune response.

Serum is tested for the production of specific antibodies, and spleen cells from animals having a positive specific antibody titer are used for cell fusions with myeloma cells to generate

hybridoma clones. Supernatants derived from hybridoma clones are tested for the presence of monoclonal antibodies having specificity against a particular validated protein (*e.g.*, SEQ ID NO:2, 4, 7, and 9, or fragments thereof). For a general description of monoclonal antibody methodology, *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988).

In addition to the baculovirus expression system, other suitable bacterial or yeast expression systems may be employed for the expression of a particular validated protein or polypeptides thereof. As with the baculovirus system, it may be advantageous to utilize one of the commercially available affinity tags to facilitate purification prior to inoculation of the animals. Thus, the a validated protein cDNA or fragment thereof may be isolated by, *e.g.*, agarose gel purification and ligated in frame with a suitable tag protein such as 6-His, glutathione-S-transferase (GST) or other such readily available affinity tag. *See, e.g., Molecular Biotechnology: Principles and Applications of Recombinant DNA*, ASM Press pp. 160-161 (*ed.* Glick, B.R. and Pasternak, J.J. 1998).

In other embodiments of the present invention, inhibitors of validated proteins are humanized anti-validated protein monoclonal antibodies. The phrase "humanized antibody" refers to an antibody derived from a non-human antibody—typically a mouse monoclonal antibody. Alternatively, a humanized antibody may be derived from a chimeric antibody that retains or substantially retains the antigen-binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans. The phrase "chimeric antibody," as used herein, refers to an antibody containing sequence derived from two different antibodies (*see, e.g.*, U.S. Patent No. 4,816,567) which typically originate from different species. Most typically, chimeric antibodies comprise human and murine antibody fragments, generally human constant and mouse variable regions.

Because humanized antibodies are far less immunogenic in humans than the parental mouse monoclonal antibodies, they can be used for the treatment of humans with far less risk of

anaphylaxis. Thus, these antibodies may be preferred in therapeutic applications that involve *in vivo* administration to a human such as, *e.g.*, use as radiation sensitizers for the treatment of neoplastic disease or use in methods to reduce the side effects of, *e.g.*, cancer therapy.

Humanized antibodies may be achieved by a variety of methods including, for example: (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as "humanizing"), or, alternatively, (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering"). In the present invention, humanized antibodies will include both "humanized" and "veneered" antibodies. These methods are disclosed in, *e.g.*, Jones et al., *Nature* (1986) *321*:522-525; Morrison et al., *Proc. Natl. Acad. Sci., U.S.A.*, (1984) *81*:6851-6855; Morrison and Oi, *Adv. Immunol.* (1988) *44*:65-92; Verhoeyer et al., *Science* (1988) *239*:1534-1536; Padlan, *Molec. Immun.* (1991) *28*:489-498; Padlan, *Molec. Immunol.* (1994) *31*(3):169-217; and Kettleborough, C.A. et al., *Protein Eng.* (1991) *4*:773-83 each of which is incorporated herein by reference.

The phrase "complementarity determining region" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. *See, e.g.,* Chothia et al., *J. Mol. Biol.* (1987) *196*:901-917; Kabat et al., U.S. Dept. of Health and Human Services NIH Publication No. 91-3242 (1991). The phrase "constant region" refers to the portion of the antibody molecule that confers effector functions. In the present invention, mouse constant regions are substituted by human constant regions. The constant regions of the subject humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu.

One method of humanizing antibodies comprises aligning the non-human heavy and light chain sequences to human heavy and light chain sequences, selecting and replacing the non-human framework with a human framework based on such alignment, molecular modeling to predict the conformation of the humanized sequence and comparing to the conformation of the

parent antibody. This process is followed by repeated back mutation of residues in the CDR region which disturb the structure of the CDRs until the predicted conformation of the humanized sequence model closely approximates the conformation of the non-human CDRs of the parent non-human antibody. Such humanized antibodies may be further derivatized to facilitate uptake and clearance, *e.g.*, via Ashwell receptors (*see, e.g.*, U.S. Patent Nos. 5,530,101 and 5,585,089, both incorporated herein by reference.

Humanized antibodies to a particular validated protein can also be produced using transgenic animals that are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and light chain loci. WO 91/10741 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin-encoding loci are substituted or inactivated. WO 96/30498 discloses the use of the Cre/Lox system to modify the immunoglobulin locus in a mammal, such as to replace all or a portion of the constant or variable region to form a modified antibody molecule. WO 94/02602 discloses non-human mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Patent No. 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy claims, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions.

Using a transgenic animal described above, an immune response can be produced to a selected antigenic molecule (*e.g.*, validated protein or fragment thereof), and antibody-producing cells can be removed from the animal and used to produce hybridomas that secrete human monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735. This publication discloses monoclonal antibodies against a variety of antigenic molecules including IL-6, IL-8, TNFα, human CD4, L-selectin, gp39, and tetanus toxin. The monoclonal

antibodies can be tested for the ability to inhibit or neutralize the biological activity or physiological effect of the corresponding protein. WO 96/33735 discloses that monoclonal antibodies against IL-8, derived from immune cells of transgenic mice immunized with IL-8, blocked IL-8-induced functions of neutrophils. Human monoclonal antibodies with specificity for the antigen used to immunize transgenic animals are also disclosed in WO 96/34096.

For purposes of the present invention, validated polypeptides and variants thereof are used to immunize a transgenic animal as described above. Monoclonal antibodies are made using methods known in the art, and the specificity of the antibodies is tested using isolated validated polypeptides. The suitability of the antibodies for clinical use is tested by, for example, exposing HIV-infected THP1 or MT-2 cells to the antibodies and measuring cell growth and/or phenotypic changes. According to the invention, inhibition of HIV-induced gene sequence expression using antisense oligonucleotides specific for validated HIV-induced polynucleotides causes an inhibition of HIV replication in THP1 and MT-2 cells. Human monoclonal antibodies specific for a particular validated protein, or for a variant or fragment thereof can be tested for their ability to inhibit HIV replication. Such antibodies would be suitable for pre-clinical and clinical trials as pharmaceutical agents for preventing or controlling HIV-mediated effects, conditions or diseases.

It will be appreciated that alternative validated protein inhibitor antibodies may be readily obtained by other methods commonly known in the art. One exemplary methodology for identifying antibodies having a high specificity for a particular validated protein is the phage display technology.

Phage display libraries for the production of high-affinity antibodies are described in, for example, Hoogenboom, H.R. et al., *Immunotechnology* (1998) *4*(1):1-20; Hoogenboom, H.R., *Trends Biotechnol.* (1997) *15*:62-70 and McGuinness, B. et al., *Nature Bio. Technol.* (1996) *14*:1149-1154 each of which is incorporated herein by reference. Among the advantages of the phage display technology is the ability to isolate antibodies of human origin that cannot

otherwise be easily isolated by conventional hybridoma technology. Furthermore, phage display antibodies may be isolated *in vitro* without relying on an animal's immune system.

Antibody phage display libraries may be accomplished, for example, by the method of McCafferty et al., *Nature* (1990) *348*:552-554 which is incorporated herein by reference. In short, the coding sequence of the antibody variable region is fused to the amino terminus of a phage minor coat protein (pIII). Expression of the antibody variable region-pIII fusion construct results in the antibody's "display" on the phage surface with the corresponding genetic material encompassed within the phage particle.

A validated protein, or fragment thereof suitable for screening a phage library may be obtained by, for example, expression in baculovirus Sf9 cells as described, *supra*. Alternatively, the validated protein coding region may be PCR amplified using primers specific to the desired region of the validated protein. As discussed above, the validated protein may be expressed in *E. coli* or yeast as a fusion with one of the commercially available affinity tags.

The resulting fusion protein may then be adsorbed to a solid matrix, *e.g.*, a tissue culture plate or bead. Phage expressing antibodies having the desired anti-validated protein binding properties may subsequently be isolated by successive panning, in the case of a solid matrix, or by affinity adsorption to a validated protein antigen column. Phage having the desired validated protein inhibitory activities may be reintroduced into bacteria by infection and propagated by standard methods known to those skilled in the art. *See* Hoogenboom, *H.R., Trends Biotechnol., supra* for a review of methods for screening for positive antibody-pIII phage.

Small Molecules and High-throughput Screening (HTS) Assays

The present invention also provides small molecule modulators of HIV infection and/or replication (or of HIV-related effects) that may be readily identified through routine application of high-throughput screening (HTS) methodologies. *Reviewed by* Persidis, A., *Nature Biotechnology* (1998) *16*:488-489. HTS methods generally permit the rapid screening of test compounds, such as small molecules, for therapeutic potential. HTS methodology employs

robotic handling of test materials, detection of positive signals and interpretation of data. Such methodologies include, *e.g.*, robotic screening technology using soluble molecules as well as cell-based systems such as the two-hybrid system described in detail above.

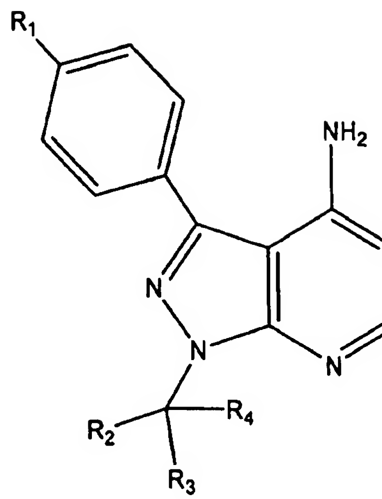
A variety of cell line-based HTS methods are available that benefit from their ease of manipulation and clinical relevance of interactions that occur within a cellular context as opposed to in solution. Test compounds are be identified via incorporation of radioactivity or through optical assays that rely on absorbance, fluorescence or luminescence as read-outs. *See, e.g.*, Gonzalez, J.E. et al., *Curr. Opin. Biotechnol.* (1998) *9*(6):624-631 incorporated herein by reference.

HTS methodology is employed, *e.g.*, to screen for test compounds that modulate or block one of the biological activities of a validated protein (*i.e.*, a protein encoded by validated HIV-induced cellular gene expression). For example, a validated protein may be immunoprecipitated from cells expressing the protein and applied to wells on an assay plate suitable for robotic screening. Individual test compounds are contacted with the immunoprecipitated protein and the effect of each test compound on an activity of the validated protein is assessed. For example, if the particular validated protein has kinase activity, the effect of a particular test compound on the kinase is assessed by, *e.g.*, incubating the corresponding immunopreciped protein in contact with the particular test compound in the presence of γ-^{32}P-ATP in a suitable buffer system, and measuring the incorporation of ^{32}P.

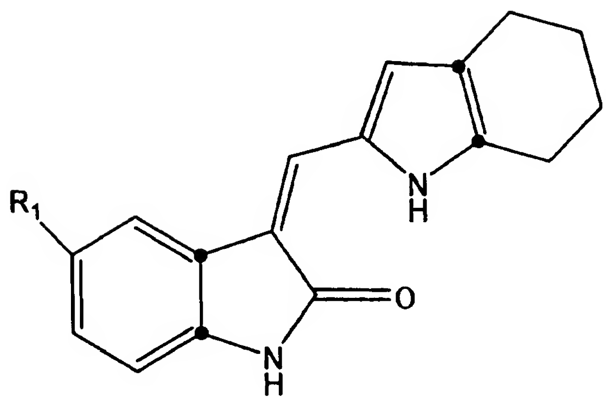
Both small molecule agonists and antagonists of particular validated proteins (SEQ ID NOS:2, 4, 7, and 9) are encompassed within the scope of the present invention.

Particular embodiments provide a method for inhibiting HIV infection and/or replication comprising administration of an *src* family kinase inhibitor selected from the group consisting of *src* family kinase-specific antisense RNA, *src* family kinase-specific siRNA, and a small molecule inhibitor of a *src* family kinase.

Preferably, the *src* family kinase is c-yes kinase (SEQ ID NOS:8 and 9). Preferably, the inhibitor is compound having the structure of Formula I, or Formula II, or salts thereof:



Formula I



Formula II

Preferably, for Formula 1, R_1 is halogen, and R_2, R_3 and R_4 are independently a C1-C3 straight or branched alkyl. Preferably, for Formula II, R_1 is $-SO_2N(CH_3)_2$, or $-SO_2NH_2$. Preferably, the inhibitor is 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Preferably, the inhibitor is 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656).

Preferably, HIV-infected THP1 or MT-2 cells are used in inventive screening assays for therapeutic compounds (see EXAMPLE 4, herein below).

<u>Methods for Assessing the Efficacy of Modulators of either HIV-induced Gene Expression or of Biological Activity Encoded thereby</u>

Inventive modulators or compounds, whether antisense molecules, siRNA, or ribozymes, proteins and/or peptides, antibodies and/or antibody fragments or small molecules, that are identified either by one of the methods described herein or via techniques that are otherwise available in the art, may be further characterized in a variety of *in vitro*, *ex vivo* and *in vivo* animal model assay systems for their ability to modulate or inhibit HIV-induced gene expression or biological activity. As discussed in further detail in the EXAMPLES 1-4 provided below, particular inventive modulators of HIV-induced gene expression are antisense inhibitors effective in reducing HIV-induced cellular gene expression levels. Thus, the present invention describes, teaches and supports methods that permit the skilled artisan to assess the effect of candidate modulators and inhibitors.

For example, candidate modulators or inhibitors of SIV-induced gene expression are tested by administration of such candidate modulators to cells that express HIV-induced genes and gene products, such as HIV-infected THP1 or MT-2 cells in the inventive HIV replication assay system. HIV-infected mammalian cells may also be engineered to express a given HIV-induced gene or recombinant reporter molecule introduced into such cells with a recombinant HIV-inducible gene plasmid construct.

Effective modulators of HIV-induced gene expression that are inhibitors will be effective in reducing the levels of HIV-induced gene mRNA as determined, *e.g.*, by Northern blot or RT-PCR analysis. For a general description of these procedures, *see, e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Press (1989) and *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, ASM Press (*ed.* Glick, B.R. and Pasternak, J.J. 1998) incorporated herein by reference. The effectiveness of a given candidate antisense molecule may be assessed by comparison with a control 'antisense' molecule (*e.g.*, a reverse complement control oligonucleotide, corresponding in orientation and size to the coding sequence complementary to the candidate antisense molecule) known to have no substantial effect on HIV-induced gene expression when administered to a mammalian cell. Exemplary control molecules include HIV-inducible gene sequence-specific reverse complement

oligonucleotides corresponding to one of the inventive antisense molecules described herein above, or to preferred representative thereof (*e.g.*, reverse complement control oligonucleotides for SEQ ID NOS:10-14).

In alternate embodiments of the present invention, the effect of modulators and inhibitors of HIV-induced gene expression on the rate of DNA synthesis after challenge with a radiation or chemotherapeutic agent may be assessed by, *e.g.*, the method of Young and Painter. *Hum. Genet.* (1989) *82*:113-117. Briefly, culture cells may be incubated in the presence of ^{14}C-thymidine prior to exposure to, *e.g.*, X-rays. Immediately after irradiation, cells are incubated for a short period prior to addition of ^{3}H-thymidine. Cells are washed, treated with perchloric acid and filtered (Whatman GF/C). The filters are rinsed with perchloric acid, 70% alcohol and then 100% ethanol; radioactivity is measured and the resulting ^{3}H/^{14}C ratios used to determine the rates of DNA synthesis.

Modulators or inhibitors of HIV-induced gene expression effective in modulating or reducing HIV-induced cellular gene expression by one or more of the methods discussed above are further characterized *in vivo* for efficacy one or more available art-recognized animal model systems (*e.g.*, SIV model). Various animal model systems for study of cancer and genetic instability associated genes are disclosed in, for example, Donehower, L.A. *Cancer Surveys* (1997) *29*:329-352 incorporated herein by reference. In particular, various art-recognized animal model systems for testing PMO antisense oligonucleotide agents, including xenograft murine models are discussed Devi, *Current Opinion in Molecular Therapeutics*, 4:138-148, 2002, incorporated by reference herein.\

Pharmaceutical Compositions

The antisense oligonucleotides and ribozymes of the present invention are synthesized by any method known in the art for ribonucleic or deoxyribonucleic nucleotides. For example, the oligonucleotides are prepared using solid-phase synthesis such as in an Applied Biosystems 380B DNA synthesizer. Final purity of the oligonucleotides is determined as is known in the art.

The antisense oligonucleotides identified using the methods of the invention modulate cancer cell proliferation, including anchorage-independent proliferation, and also modulate HIV-mediated phenotypic changes, including spindle formation.

Therefore, pharmaceutical compositions and methods are provided for interfering with HIV infection and/or replication, or for HIV-related conditions or diseases, comprising contacting tissues or cells with one or more of antisense oligonucleotides or siRNA identified using the methods of the invention. Preferably, an antisense oligonucleotide having one of SEQ ID NOS:10-14, or preferably SEQ ID NOS:10-13, is administered. Preferably, the antisense oligonucleotide is a PMO antisense oligomer (PMO).

The methods and compositions may also be used to treat other HIV-associated conditions and disorders known in the art.

The invention provides pharmaceutical compositions of antisense oligonucleotides, siRNA and ribozymes complementary to validated HIV-induced cellular gene mRNA gene sequences, corresponding to SEQ ID NOS:1, 3, 5, 6, and 8, and complements thereof as active ingredients for therapeutic application. These compositions can also be used in the methods of the present invention. Where required the compounds are nuclease resistant. In general the pharmaceutical composition for modulating HIV-mediated cellular proliferation or phenotype in a mammal includes an effective amount of at least one antisense oligonucleotide (or siRNA agent, etc) as described above needed for the practice of the invention, or a fragment thereof shown to have the same effect, and a pharmaceutically physiologically acceptable carrier or diluent.

Particular embodiments provide a method for reducing HIV infection and/or replication in a subject comprising administering an amount of an antisense oligonucleotide (or siRNA agent) of the invention effective to reduce said HIV infection and/or replication Preferably the antisense oligomer (siRNA) is based on one of SEQ ID NOS:1, 3, 5, 6, and 8. More preferably the antisense oligonucleotide is one of SEQ ID NOS:10-13.

The pharmaceutical composition for inhibiting HIV infection and/or replication in cells in a mammal consists of an effective amount of at least one active ingredient selected from siRNA agents, or antisense oligonucleotides complementary to the HIV-induced cellular gene mRNA, including the entire HIV-induced gene mRNA or having shorter sequences as set forth in SEQ ID

NOS:15-21, and a pharmaceutically acceptable carrier or diluent. Combinations of the active ingredients are contemplated and encompassed within the scope of the invention.

The compositions can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques as required by the cells being treated. For delivery within the CNS, intrathecal delivery can be used with for example an Ommaya reservoir or other methods known in the art. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention. Cationic lipids may also be included in the composition to facilitate oligonucleotide uptake. Implants of the compounds are also useful. In general, the pharmaceutical compositions are sterile.

In the method of the present invention, HIV-related cells are contacted with an efficacious amount of the bioactive antisense oligonucleotide (or siRNA agent) for the HIV-induced cellular gene mRNA or a fragment thereof shown to have substantially the same effect. In an embodiment, the mammal to be treated is human but other mammalian species can be treated in veterinary applications.

Bioactivity, relating to a particular oligonucleotide modulator, refers to biological activity in the cell when the oligonucleotide modulator is delivered directly to the cell and/or is expressed by an appropriate promotor and active when delivered to the cell in a vector as described below. Nuclease resistance of particular modulators is provided by any method known in the art that does not substantially interfere with biological activity as described herein.

Significantly, PMO chemistry is not RNase H competent (*discussed in* Devi, *Current Opinion in Molecular Therapeutics*, 4:138-148, 2002).

"Contacting the cell" refers to methods of exposing, delivery to, or 'loading' of a cell of antisense oligonucleotides whether directly or by viral or non-viral vectors, and where the antisense oligonucleotide is bioactive upon delivery. The method of delivery will be chosen for the particular cell type being treated. Parameters that affect delivery can include the cell type affected and its location as is known in the medical art.

The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated. It is noted that humans are treated generally longer than the Examples exemplified herein, which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses as determined by the medical practitioners and treatment courses will be repeated as necessary until diminution of the disease is achieved. Optimal dosing schedules may be calculated using measurements of drug accumulation in the body. Practitioners of ordinary skill in the art can readily determine optimum dosages, dosing methodologies, and repetition rates. Optimum dosages may vary depending on the relative potency of the antisense oligonucleotide (or siRNA agent), and can generally be determined based on values in *in vitro* and *in vivo* animal studies and clinical trials. Variations in the embodiments used may also be utilized. The amount must be effective to achieve improvement including but not limited to decreased infection, viral replication, or to improved survival rate or length or decreased drug resistance or other indicators as are selected as appropriate measures by those skilled in the art.

Although particular inventive antisense oligonucleotides (or siRNA agents) may not completely abolish HIV infection and/or replication, or other HIV-induced effects *in vitro*, as exemplified herein, these antisense compounds and agents are nonetheless clinically useful where they inhibit HIV-related infection, and/or replication, etc., enough to allow complementary treatments, such as chemotherapy, radiation therapy, or other drug therapies to be effective or more effective. The pharmaceutical compositions of the present invention therefore are administered singly or in combination with other drugs, such as HIV inhibitory agents (AZT, etc.), cytotoxic agents, immunotoxins, alkylating agents, anti-metabolites, antitumor antibiotics and other anti-cancer drugs and treatment modalities that are known in the art.

Cocktails of antisense inhibitors directed against several HIV-induced gene sequences are contemplated and within the scope of the present invention.

The composition is administered and dosed in accordance with good medical practice taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for HIV inhibition is thus determined by such considerations as are

known in the art. The pharmaceutical composition may contain more than one embodiment or modulator of the present invention.

The nucleotide sequences of the present invention can be delivered either directly or with viral or non-viral vectors. When delivered directly the sequences are generally rendered nuclease resistant. Alternatively, the sequences can be incorporated into expression cassettes or constructs such that the sequence is expressed in the cell. Generally, the construct contains the proper regulatory sequence or promoter to allow the sequence to be expressed in the targeted cell.

Once the oligonucleotide sequences are ready for delivery, they can be introduced into cells as is known in the art (*see, e.g.*, Devi, *Current Opinion in Molecular Therapeutics*, 4:138-148, 2002). Transfection, electroporation, fusion, liposomes, colloidal polymeric particles and viral vectors as well as other means known in the art may be used to deliver the oligonucleotide sequences to the cell. The method selected will depend at least on the cells to be treated and the location of the cells and will be known to those skilled in the art. Localization can be achieved by liposomes, having specific markers on the surface for directing the liposome, by having injection directly into the tissue containing the target cells, by having depot associated in spatial proximity with the target cells, specific receptor mediated uptake, viral vectors, or the like.

Administration and clinical dosing of PMO antisense therapeutic agents is discussed, for example, in Devi, *supra*, and in Arora et al. *Journal of Pharmaceutical Sciences*, 91:1009-1018, 2001, both incorporated by reference herein.

The present invention provides vectors comprising an expression control sequence operatively linked to the oligonucleotide sequences of the invention. The present invention further provides host cells, selected from suitable eukaryotic and prokaryotic cells, which are transformed with these vectors as necessary. Such transformed cells allow the study of the function and the regulation of malignancy and the treatment therapy of the present invention.

Vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the oligonucleotides in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors.

Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, liposomes and other recombination vectors. The vectors can also contain elements for use in either prokaryotic or eukaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor, Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et al., *BioTechniques* (1986) *4*:504-512 and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors.

Recombinant methods known in the art can also be used to achieve the antisense inhibition (or siRNA mediated inhibition) of a validated target nucleic acid. For example, vectors containing antisense nucleic acids can be employed to express an antisense message to reduce the expression of the validated target nucleic acid and therefore its activity.

The present invention also provides a method of evaluating if a compound inhibits transcription or translation of an HIV-induced cellular gene sequence, and thereby modulates (*i.e.*, reduces) viral infection, replication, cell proliferation or phenotypic differentiation, comprising transfecting a cell with an expression vector comprising a nucleic acid sequence encoding a HIV-induced cellular gene sequence, the necessary elements for the transcription or translation of the nucleic acid; administering a test compound; and comparing the level of expression of the HIV-induced cellular gene sequence with the level obtained with a control in the absence of the test compound. Alternatively, as is shown in the Examples herein, such an expression vector is not required, and test compounds are administered to HIV-infected cells, such as HIV-infected THP1 or MT-2 cells.

The present invention provides detectably labeled oligonucleotides for imaging HIV-induced cellular gene sequences (polynucleotides) within a cell. Such oligonucleotides are useful for determining if gene amplification has occurred, for assaying the expression levels in a

cell or tissue using, for example, *in situ* hybridization as is known in the art, and for diagnostic and/or prognostic purposes.

Diagnostic and/or Prognostic Assays for HIV and HIV-related conditions or diseases

The present invention provides for diagnostic and/or prognostic cancer assays based on differential measurement of validated HIV-induced gene expression. Preferred validated HIV-induced gene sequences are represented herein by SEQ ID NOS:1, 3, 5, 6, and 8, and complements thereof, along with the corresponding gene products SEQ ID NOS:2, 4, 7 and 9, and combinations thereof.

Typically, such assays involve obtaining a tissue sample from a test tissue, performing an assay to measure expression of at least one validated HIV-induced gene product (*e.g.*, mRNA or protein encoded thereby) derived from the tissue sample, relative to a control sample, and making a diagnosis or prognosis based, at least in part, thereon.

In particular embodiments the present inventive oligomers, such as those based on validated SEQ ID NOS:1, 3, 5, 6, and 8, and complements thereof, or preferably SEQ ID NOS:10-13, or arrays comprising any of the preceding validated sequences or gene products, as well as a kit based thereon are useful for the diagnosis and/or prognosis of HIV infection and/or replication, or other HIV-related cell disorders, conditions or diseases.

The present invention moreover relates to a method for manufacturing a diagnostic agent and/or therapeutic agent for HIV diagnosis and/or therapy of HIV-related conditions or diseases, the diagnostic agent and/or therapeutic agent being characterized in that at least one inventive validated modulator of HIV-induced gene expression is used for manufacturing it, possibly together with suitable additives and ancillary agents.

Diagnostic kits are also contemplated, comprising at least one primer and/or probe specific for a validated HIV-induced cellular gene sequence according to the present invention, possibly together with suitable additives and ancillary agents.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the invention.

EXAMPLE 1

(HIV-infected THP1 and MT-2 cells are a valid *in vivo* model system for HIV replication)

The HIV-1 strain used in the model system was the 89.6 strain. This is a dual tropic (X4/R5) HIV strain, meaning that it can infect cells utilizing CD4 and either the CXCR4 or the CCR5 co-receptor. Thus, both T cells (*e.g.*, MT-2) and macrophages (*e.g.*, THP-1) are susceptible to infection by the same virus strain. HIV-1 89.6 was originally provided by the investigator who isolated and characterized it, Dr Ronald Collman (Collman et al, *J. Virology* 66:7517, 1992). Applicant's expanded the virus by culture in PBMC, and concentrated it for use in the inventive system as described in EXAMPLE 2, herein below.

HIV-infected THP-1 and MT-2 cells. The cell lines selected for use herein include the monocyte line THP-1 and the T cell leukemia cell line MT-2.

The THP-1 cell line is CD4+, highly permissive for HIV infection, and has been used by numerous investigators for studying various aspects of HIV biology, either prior to or in concert with examination of primary cells. Reference to the HIV literature over the past 6 months reveals several studies of HIV biology and therapeutics that underscore this point: Briquet & Vaquero, *Virology* 292:177-84, 2002 (Immunolocalization studies of an antisense protein in HIV-1-infected cells and viral particles); Branch, D. R. et al.. *Aids* 16:309-19, 2002 (VPAC1 is a cellular neuroendocrine receptor expressed on T cells that actively facilitates productive HIV-1 infection); Nguyen & Taub,. *J Immunol* 168:4121-6 (CXCR4 function requires membrane cholesterol: implications for HIV infection); Ho, W. Z., et al., *Faseb J* 16:616-8, 2002 (HIV enhances substance P expression in human immune cells); Hayes, M. M., et al.,. *J Biol Chem* 277:16913-9, 2002 (Peroxisome proliferator-activated receptor gamma agonists inhibit HIV-1 replication in macrophages by transcriptional and post-transcriptional effects); Lenardo, M. J. et al. *J Virol* 76:5082-93, 2002 (Cytopathic killing of peripheral blood CD4(+) T lymphocytes by human immunodeficiency virus type 1 appears necrotic rather than apoptotic and does not require env.); Bolton, D. L. et al.. *J Virol* 76:5094-107, 2002 (Death of CD4(+) T-cell lines

caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis); Alfano, M., et al., *Proc Natl Acad Sci U S A* 99:8862-7, 2002 (Urokinase-urokinase receptor interaction mediates an inhibitory signal for HIV-1 replication); Wu, L., Martin, et al., *J Virol* 76:5905-14, 2002 (Functional evaluation of DC-SIGN monoclonal antibodies reveals DC-SIGN interactions with ICAM-3 do not promote human immunodeficiency virus type 1 transmission); Mautino & Morgan, *Hum Gene Ther* 13:1027-37, 2002 (Enhanced inhibition of human immunodeficiency virus type 1 replication by novel lentiviral vectors expressing human immunodeficiency virus type 1 envelope antisense RNA).

Reduced biological noise and the capacity to infect the majority of cells in culture are both important details for a relevant array-based analysis of the effect of HIV infection, and both of these parameters have been achieved by the use of this CD4+ cell line. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA). They are currently available for our use as multiple age- and passage-matched cryopreserved aliquots. All cell lines are maintained as suspension cultures in vented T75 tissue culture flasks at densities of 5×10^6 to 5×10^7 cells/ml in RPMI supplemented with 2mM glutamine, penicillin/streptomycin and 10% FBS. Following treatment with TPA, THP-1 cells differentiate into adherent macrophage-like cells and are cultured in 60mm tissue culture-treated dishes.

Additionally, the T cell line "MT-2" was used. MT-2 is a human T cell leukemia cell line that can be grown in suspension and, like THP-1 cells, are very susceptible to acute infection with HIV. The cells can be efficiently loaded with antisense oligonucleotides. In addition, they have been shown by other investigators to provide a sensitive and reproducible system to test antiviral agents (*see, e.g.*, Haertle et al, *J. Biol. Chem.* 263:5870-5875, 1988). MT-2 cells are available through the NIH AIDS Research and Reference Reagent Program.

HIV-infected THP-1 cells. HIV-infected THP-1 cells were used as an *in vitro* model for examining cellular gene expression during the HIV replication cycle. The HIV-1 89.6 was initially grown in PBMC, and concentrated using Amicon membranes prior to use for infection of THP-1 cells. Briefly, as the goal of the present studies was to profile cellular gene expression

over the course of a complete viral replication cycle, the protocol called for synchronous infection of the majority of cells in culture, with multiple samplings over the first 48 hours post-infection (PI). Since the number of target cells required to yield sufficient RNA for gene profiling at multiple times PI is high ($>10^8$), synchronous infection of such cell numbers requires high titer virus stocks. However, virus titers derived from PBMC supernatants are typically only in the region of $1x10^5$ to $1x10^6$ pfu/ml. Additionally, such supernatants may also contain cellular growth factors with the capacity to influence gene expression independent of virus-induced effects.

To circumvent these obstacles, PBMC-derived stocks of HIV-1 89.6 were concentrated 20-fold using Amicon Ultra-15 centrifugal filter devices (Millipore, Bedford MA). This procedure yielded high titer virus (± $1x10^7$ pfu/ml) that could be resuspended in medium free from host-cell derived factors.

The potency of concentrated virus stocks is exemplified in FIGURE 1 which illustrates MAGI cells (HeLa-$CD4^+$-HIV LTR-ß-gal) infected with 1 µl or 0.1 µl of concentrated virus as compared to 1µl of unconcentrated virus. MAGI cells infected with 0.1µl of unconcentrated 89.6 exhibited no sign of infection. MAGI cells are Hela CD4 cells stably transfected with the β-galactosidase gene under the control of the HIV LTR. When MAGI cells are productively infected with HIV, β-galactosidase expression is induced by tat-transactivation and the number of blue cells revealed by staining is a measure of virus titer.

The left and center panels of FIGURE 1 show the number of blue-staining cells increasing in a dose responsive manner with the use of concentrated HIV-1 89.6 stocks.

EXAMPLE 2

(Nucleic acid microarray technology was used for gene expression profiling of HIV-infected THP-1 cells to identify cellular genes whose expression is regulated by HIV)

Nucleic Acid Microarray Data Analysis. Cellular genes involved in HIV-1 replication were identified by using DNA microarrays to examine the differential gene expression profiles of THP-1 cells before and after HIV-infection.

For RNA isolation and fluorescent labeling, two RNA probe samples from THP-1 cells, independently infected with KSHV, and two independent uninfected RNA probe samples were prepared. Briefly, THP1 monocytes infected with HIV isolate MN or with 89.6 were harvested at 2, 4, 6, 8, 10, and 12 hours post infection (PI). Uninfected cells were harvested in parallel.

Generally, RNA was isolated using the RNeasy™ RNA isolation kit (QIAGEN Inc., Valencia, CA). After DNase treatment and another round of RNeasy purification, labeled cDNA was prepared as described previously (*see* Salunga et al., *In* M. Schena (ed.), DNA microarrays. A practical approach; Oxford Press, Oxford, United Kingdom, 1999; *and see* Simmen et al., *Proc. Natl. Acad. Sci. USA* 98:7140-7145, 2001). Briefly, double-stranded cDNA was selectively synthesized from the RNA samples. Biotin-labeled cRNA was produced from the cDNA by *in vitro* transcription (IVT) using methods well known in the art.

For expression profile screening, the biotin-labled cRNA probe preparations were fragmented and hybridized to Affymetrix (Santa Clara, CA) U133A and U133B arrays or to U95A arrays (Affymetrix U133A, U133B and U95A GeneChip® arrays). The Human Genome U133 (HG-U133) set, consists of two GeneChip® arrays, and contains almost 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes (Affymetrix technical information). The set design uses sequences selected from GenBank®, dbEST, and RefSeq (*Id*).

The Affymetrix GeneChip® platform was chosen for these studies as it is the industry leader in terms of array content, platform stability and data quality. Images of the arrays were analyzed using the Affymetrix microarray analysis suite software, MAS. This software package is used for converting images to raw numerical data, and direct comparisons between control and experimental samples. When making such comparisons, MAS provides robust statistical algorithms for determining changes in expression between the two samples, along with p-values

and confidence limits on such changes. For each probe set, MAS records whether there was no change, increased expression or decreased expression. To determine if the number of gene expression changes in common between two or more experiments is significant, we routinely compared the number of genes in such lists to the number expected if the experiments were independent.

Each of the THP-1 infected/uninfected sample comparisons resulted in multiple probe sets with increased expression, with some sets showing increased expression in both infections. Increased or decreased expression was based on 'calls' from MAS software which typically corresponds to about a two-fold change.

Genes with increased transcription in at least two adjacent time points across the two infections were considered for possible validation studies using PMOs. Genes with evidence of induction due to interferon, in separate experiments conducted in endothelial cells were excluded from consideration. Annotation from the Gene Ontology consortium was compiled for the remaining genes. Those without informative annotation were dropped from further consideration.

Approximately 20 genes were selected from the remaining list for testing as potential anti-viral targets with PMO-AS.

Representative microarray expression data. TABLE 1 (see herein, next page) shows expression data obtained according to the present invention for the HMG20B, HRH1, NP, YES and ARF1 gene sequences using Affymetrix U133 arrays as indicated. Expression is presented as "fold-increase" in signal for two to four independent infected/mock infected comparisons, as described herein above.

TABLE 1. U133 microarray expression data for particular KSHV-induced gene sequences.

GENE	ARRAY	Affymetrix Probe Set	Fold Increase T89602 x 067TMK 2hrs	Fold Increase T89604 x 067TMK 4hrs	Fold Increase T89606 x 067TMK 6hrs	Fold Increase T89608 x 067TMK 8hrs	FC_T89610 x 067TMK 10	FC_T89612 x 067TMK 12
HMG20B	UI33A	209113_s_at	11.31	3.03	4.29	6.06	-1.52	-1.74
HRH1	UI33A	205579_at	2.64	2.64	2	1.74	1.52	1.87
HRH1	UI33A	205579_at	2.64	2.64	2	1.74	1.52	1.87
NP	UI33A	201695_s_at	1.87	2.14	2.64	3.03	2.3	1.52
NP	UI33A	201695_s_at	1.87	2.14	2.64	3.03	2.3	1.52
YES	U133A	202932_at	1	1.41	1.32	1.15	1.52	2
YES	U133A	202933_s_at	1.07	1.15	-1.07	-1.07	1.74	1.32
ARF1	U133A	208750_s_at	6.06	4	2.46	6.06	1.07	1.07
ARF1	U133A	208750_s_at	6.06	4	2.46	6.06	1.07	1.07

EXAMPLE 3

(Target validation; genes necessary for virally-induced morphological changes in HIV-infected THP-1 and MT-2 cells were identified using antisense PMOs)

Antisense Phosphorodiamidate Morpholino Oligomers (PMOs). PMOs (see, *e.g.*, Summerton, et al., *Antisense Nucleic Acid Drug Dev.* 7:63-70, 1997; and Summerton & Weller, *Antisense Nucleic Acid Drug Dev.* 7:187-95, 1997) are a class of antisense drugs developed for treating various diseases, including cancer. For example, Arora et al. (*J. Pharmaceutical Sciences* 91:1009-1018, 2002) demonstrated that oral administration of *c-myc*-specific and CYP3A2-specific PMOs inhibited *c-myc* and CYP3A2 gene expression, respectively, in rat liver by an antisense mechanism of action. Likewise, Devi G.R. (*Current Opinion in Molecular Therapeutics* 4:138-148, 2002) discusses treatment of prostate cancer with various PMO therapeutic agents). See also recent reviews by Milhavet et al., and by Gitlin et al (Milhavet et al *Pharmacological Reviews* 55:629-648, 2003; and Gitlin et al., *J. Virol.* 77:7159-7165, 2003; incorporated herein by reference).

PMOs were designed and used, according to the present invention to silence genes identified as being consistently up-regulated in HIV-infected THP-1 and MT-2 cells. PMO-AS (PMO antisense) are about 15-18 base pair oligonucleotides complementary to a specific mRNA start codon that prevent message translation through steric hindrance at the ribosome binding site (Ghosh, et al. *Methods in Enzymology* 313:135-143, 2000). PMOs do not activate RNAse H, (*Id*). Typically, it is preferable and sufficient to target the region of the start codon to block translation, but, as discussed herein above, other mRNA regions, both coding and non-coding can be effectively targeted according to the present invention.

Antisense Gene Silencing using PMOs. Genes identified as being consistently up-regulated in HIV-infected THP-1 cells in the above described nucleic acid microarray/gene expression profiling experiments were further analyzed to identify those necessary for viral replication. Silencing of such genes substantially reduced HIV replication relative to controls, as measured by p24 gag ELISA assays (*see* TABLE 2 below), and validated these cellular gene

targets for respective therapeutic methods and compositions for blocking HIV replication, and thus HIV-related conditions and diseases.

Introduction of antisense PMO into HIV-infected THP-1 or MT-2 cells. Antisense PMO molecules, for delivery purposes, are typically converted to a paired duplex together with a *partially* complementary cDNA oligonucleotide in the weakly basic delivery reagent ethoxylated polyethylenimine (EPEI) (Summerton, *supra*). The anionic complex binds to the cell surface, is taken up by endocytosis and eventually released into the cytosol. A protocol for optimum uptake of antisense PMO in THP-1 and MT-2 cells was developed using a modification of the EPEI method. PMO-AS were obtained from GeneTools (Genetools, LLC, One Summerton Way, Philomath, OR 97370).

Briefly, loading into target cells was accomplished by complexing the PMO with a proprietary loading reagent, Ethoxylated Polyethylenimine (EPEI). Target cells (MT-2 or THP-1) were introduced into snap cap conical tubes ($5x10^5$ cells/tube) in 900 μl serum free RPMI and the EPEI-PMO complex was added for 4 hours at 37°C in a 7% CO_2 atmosphere. The PMO-EPEI complex was prepared by diluting 5 μl PMO in 40 μl sterile RNase-free water, adding 5 μl EPEI, vortexing and incubating the sample for 20 minutes at room temperature. The sample was then mixed with 50 μl serum-free RPMI and added to the target cells to yield a final volume of 1ml. At the end of the loading period, cells were gently pelleted and the PMO solution was removed by aspiration. After a further wash, cells were incubated in complete RPMI for 12 hrs prior to HIV infection to allow for recovery and initiation of PMO action.

HIV-1 Infection. PMO-treated THP-1 or MT-2 cells were infected with 50 μl of concentrated HIV-1 89.6 diluted to 1 ml in serum-free RPMI. The following control cultures were identically infected: (i) to control for any non-specific effect of the loading protocol, cells exposed to EPEI alone during the loading procedure; (ii) as a positive control for normal HIV infection, cells that were not exposed to either EPEI or EPEI-PMO; (iii) as a control for HIV inhibition, cells infected with HIV in the presence of 2 μM AZT and maintained in AZT throughout.

Cells were exposed to HIV for 4 hours. The inoculum was removed by 3 cycles of pelleting and rinsing. The cells were resuspended in 1.5 ml of complete RPMI and 0.5 ml immediately removed at Time 0 to leave a final volume of 1 ml. The Time 0 sample served as a measure of residual virus. At each harvest time (T1 through T4), 0.5 ml of supernatant was removed and replaced with an equal volume of complete RPMI. Samples were stored at -80°C until evaluation using a p24 ELISA assay (described below). Each experimental variable and control was performed in duplicate. T1 through T4 typically corresponded to 16, 24, 40 and 48 hrs PI, respectively.

Cellular distribution of introduced FITC-labeled POM antisense molecules. To ensure the success of PMO loading and HIV infection for each assay, cells cultured in 35mm tissue culture plates were loaded with a FITC tagged PMO, or infected with HIV, and monitored microscopically. Figure 2a (left panel) illustrates a representative fluorescent image of FITC-labeled PMO antisense uptake by MT-2 cells; that is, successful delivery of a FITC-tagged PMO to MT-2 cells. The right panel show results with the EPEI only control. Therefore, the introduced PMO antisense oligomers were readily taken up by MT-2 cells, distributed within the cytosol, and determined to be stable over the relevant time periods in MT-2 cells.

Figure 2B illustrates a typical HIV control (no PMO AS) where extensive HIV-induced syncytia are seen in HIV infected MT-2 at 48 hrs PI.

Validation of KSHV-induced gene sequences; HIV-1 p24 antigen ELISA. To monitor the effect of PMO-targeting of cellular proteins on HIV replication, supernatants harvested from PMO-treated and control THP and MT-2 cells were assayed using an HIV p24 antigen ELISA. The rationale behind this assay is described in the following paragraph.

The HIV-1 gag protein p24 is the major internal structural component of the virion core. The Coulter p24 assay is an enzyme-liked immunosorbent assay (ELISA) performed using a commercially available kit (Beckman Coulter). It was developed specifically for the detection and quantitation of HIV-1 p24 in plasma or serum, for clinical purposes, or in tissue culture

supernatants, to monitor virus replication. Each kit contains a 96-well microtiter tray pre-coated with a monoclonal antibody to p24. A specimen of plasma, serum or tissue culture supernatant is added to each well along with a viral lysis buffer and incubated to allow any p24 antigen present to bind to the coated well. Following a wash, biotinylated human anti-HIV IgG is added and incubated to allow complexing to any bound p24. Following another wash, strepatavidin-HRP is added to complex with any bound biotinylated complexes. A substrate reagent is added to form a blue color upon reaction with HRP, the reaction is stopped with acid, and the absorbance measured spectrophotometrically. The intensity of color development is directly proportional to the amount of p24 present in the test sample. For each assay, a series of wells are devoted to running a standard curve that utilizies known amounts of purified p24 antigen. The standard curve is used to monitor assay performance and to qualitatively determine the amount of p24 (in pg/ml) in the test sample.

The levels of p24 measured in supernatants from PMO-treated and control THP-1 and MT-2 cells were plotted graphically as p24 production (y-axis) versus time PI (x-axis). Typical results for THP-1 cells and MT-2 cells are graphically depicted in Figure 3 A and B respectively.

Figure 3A shows inhibition curves of HIV replication in HIV-infected THP-1 cells (human myeloid (monocyte/macrophage) cell line derived from an acute monocytic leukemia) by PMOs specific for particular HIV-induced cellular genes as follows: upper filled diamonds correspond to no PMO control; triangles correspond to TNIP; lower filled diamonds correspond to c-YES; dark "X"s correspond to HRH1; light "X"s correspond to NP; filled squares correspond to HMG20; and vertical lines correspond to AZT control. The HIV-1 P24 ELISA assay monitors HIV p24 gag production (in pg/ml along the y-axis) by PMO-treated HIV-infected THP-1 cells. The x-axis shows time ("T1-T4" typically correspond to 16, 24, 40 and 48 hrs PI). PMOs corresponding to HMG20, HRH-1, NP and c-YES were particularly effective at reproducibly inhibiting HIV replication.

Figure 3B shows inhibition curves of HIV replication in HIV-infected MT-2 cells (human T cell leukemia cell line) by PMOs specific for particular HIV-induced cellular genes as follows:

upper curve filled diamonds correspond to EPEI (ethoxylated polyethylenimine); open squares correspond to HIV only; open triangles correspond to ARF; filled triangles correspond to NP; lower curve filled diamonds correspond to HMG20; "X"s correspond to c-YES; filled squares correspond to HRH-1; and vertical lines correspond to HIV plus AZT control. The HIV-1 P24 ELISA assay monitors HIV p24 gag production (in pg/ml along the y-axis) by PMO-treated HIV-infected MT-2 cells. The x-axis shows time ("T1-T4" typically correspond to 16, 24, 40 and 48 hrs PI). As in the case of HIV-infected THP-1 cells (Figure 3A), PMOs corresponding to HMG20, HRH-1, NP and c-YES were particularly effective at reproducibly inhibiting HIV replication.

Systematic testing of PMOs in this system led to the identification of 4 PMOs that reproducibly inhibited HIV replication in both the monocyte and the T cell line. These PMOs were designed to inhibit expression of the following cellular proteins: HMG20, HRH-1, NP and c-Yes.

TABLE 2 shows the validation results for five induced genes identified in the experiments of EXAMPLE 2 herein above. For four of the induced genes, suppression by sequence-specific PMO antisense oligonucleotides led to substantial inhibitory effects (either full or intermediate inhibition) on HIV replication, as measured by p24 gag production: HMG20B (*homo sapiens* high-mobility group 20B, accession number NM_006339, and known variants); HRH1 (*homo sapiens* histamine receptor H1, accession numbers NM_00861 and BC060802, and known variants); NP (*homo sapiens* nucleoside phosphorylase, accession number NM_000270, and known variants); and YES1 (*homo sapiens* v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1, accession number NM_005433, and known variants). The specific PMO antisense oligomers used in these experiments for silencing the HIV-induced gene sequences are also shown in TABLE 2, along with corresponding SEQ ID NOS.

TABLE 2. Validated Gene Targets; suppression (silencing) of particular HIV-1 89.6-induced genes substantially inhibited HIV replication as measured by gag 24 production.

GENE	PMO Antisense Sequence (5' to 3')	Extent of PMO-induced Inhibition of HIV replication
HMG20B	CGCCCAGCATCTTGGTGATCTCGGG	positive
HRH1	GCGAAAGAGCAGCCGCCAGTTATGG	positive
NP	CTTCATAGGTGTATCCGTTCTCCAT	positive
YES	TTTCTTTACTTTTAATGCAGCCCAT	positive
ARF1	ATGCTTGTGGACAGGTGGAAGGACA	(negative)

TABLE 3 summarizes GenBank mRNA and EST accession numbers for particular HIV-induced genes, including for the four validated gene sequences listed in TABLE 2. Gene names, Unigene clusters, and GenBank accession numbers are as assigned by the National Center for Biotechnology Information (NCBI), and are incorporated by reference herein, including splice and allelic variants of mRNA sequences.

TABLE 3. GenBank accession numbers for particular HIV-induced genes, including for the HMG20B, HRH1, NP, and YES1 gene sequences validated herein.

GENE	Unigene Cluster	Accession Numbers; mRNAs	Accession Numbers; ESTs
HMG20B	Hs.406534	AF072836.1, NM_006339.1, AL355709.1, AL355691.1, AL355703.1, AF146223.1, AF072165.1, AF288679.1, BC003505.1, BC002552.1, AF331191.1, BC004408.1, BC021585.1, AK090733.1, AF318366.1, AL355698.1, AL355702.1	BE379548.1, BG768730.1, BG768163.1, BG768132.1, BG767749.1, BE387852.1, BE387970.1, BE388514.1, BE388977.1, BG767740.1
HRH1	Hs.1570	NM_000861.2, D28481.1, AY136743.1, Z34897.1, AF026261.1	CB960117.1, BI333356.1, AI925675.1, AI565677.1, AI952059.1, AI889049.1, AI926322.1, AA613545.1, AA582697.1, AI954670.1
NP	Hs.75514	NM_000270.1, AK098544.1, AF116670.1, X00737.1	BG032220.1,BG177426.1, BG429168.1, BG500365.1, BG527082.1, BG831409.1, BI087530.1, BI225123.1, BI225577.1, BI197180.1
YES	Hs.194148	BC048960.1, NM_005433.2, BC031080.1, M15990.1	BQ932574.1, BI861691.1, BI560649.1, BI548150.1, AI799358.1, AI367561.1, AA604737.1, AI445609.1, R28423.1, R25397.1

HIV-induced genes excluded as therapeutic targets by PMO antisense validation protocol. The above Examples show that with respect to particular identified HIV-induced genes (*e.g.*, ARF1), treatment of HIV-infected MT-2 with the respective antisense PMO oligonucleotides had little or no affect on HIV replication (p24 gag production) despite effectiveness of such antisense agents in mediating silencing of the respective gene sequences. This was not unexpected, because HIV-related modulation of some cellular genes would reasonably be expected to be either ancillary to, or downstream from the regulatory cascades involved in HIV replication.

Significantly, the identification of HIV-induced gene sequences which, upon silencing, have no effect on HIV replication provides internal confirmation (apart from the use of particular control PMO antisense molecules, etc.) that the inventive gene-silencing mediated inhibition of HIV replication is not mediated through ancillary or non-sequence-specific secondary effects of the respective PMO antisense molecules.

Therefore, data presented herein describes, teaches and supports the use of sequence-specific PMO antisense oligomers, *inter alia*, for (i) validation of therapeutic 'targets'; that is, for identification of HIV-induced cellular gene products *required* for HIV-induced cellular phenomena (*e.g.* replication, etc.), and (ii) as effective, non-toxic inhibitors of such validated therapeutic targets for modulation of HIV infection and treatment of HIV-related disorders and diseases. This utility is especially valuable where the particular gene products otherwise lack suitable art-recognized small molecule inhibitors.

Additionally, in view of deficiencies in the prior art teachings, these data emphasize the significance of *functional validation* of HIV-induced gene sequences, according to the present invention to provide targets, compositions and methods having utility for blocking HIV infection and replication, and for treating HIV-related conditions and diseases.

EXAMPLE 4

(The *src* family kinase inhibitor PP2 inhibits HIV replication in MT-2 cells)

As discussed herein, particular embodiments of the present invention provide screening assays for identification of compounds useful to modulate HIV infection, comprising: contacting

HIV-infected cells with a test agent; measuring, using a suitable assay, expression of at least one validated HIV-induced cellular gene sequence; and determining whether the test agent inhibits said validated gene expression relative to control cells not contacted with the test agent, whereby agents that inhibit said validated gene expression are identified as compounds useful to modulate HIV infection and/or replication.

Preferably, the at least one validated HIV-induced cellular gene sequence is selected from the cDNA and protein sequence group consisting of HMG20B, HRH1, NP and c-YES, and combinations thereof (*i.e.*, consisting of SEQ ID NOS:1-9). Preferably, expression of at least one validated HIV-induced cellular gene sequence is expression of mRNA, or expression of the protein encoded thereby. Preferably, agents that inhibit said validated gene expression are further tested for the ability to modulate HIV-mediated effects on cellular proliferation and/or phenotype.

As shown in FIGURE 4, addition of the *src* family kinase inhibitor PP2 to HIV-infected MT-2 cells resulted in substantial decrease in HIV p24 production. Specifically, FIGURE 4 shows inhibition of HIV p24 production in MT-2 cells infected with HIV 89.6 in the continued presence (10 μM) of the src family kinase inhibitor PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; *e.g.*, Calbiochem; catalog no. 529573). PP3, and DMSO correspond to inactive analog, and carrier control, respectively. AZT inhibition is also included as a positive control. Time 0 is immediately post-infection, whereas times 1, 2 and 3 correspond to 24, 48, and 72 hrs PI, respectively. The curves are as follows: upper closed diamonds correspond to HIV alone; lower curve closed diamonds correspond to PP2; filled squares correspond to PP3; filled triangles correspond to DMSO; and "X"s correspond to AZT. The HIV-1 P24 ELISA assay monitors HIV p24 gag production by the various treated HIV-infected MT-2 cells.

The ability to substantially reduce HIV replication through specific inhibition of src family kinase c-YES activity further demonstrates a critical role for src family kinase signaling

in HIV replication in MT-2 and THP-1 cells and further supports a role for upregulation of c-YES as a factor in HIV replication and related events.

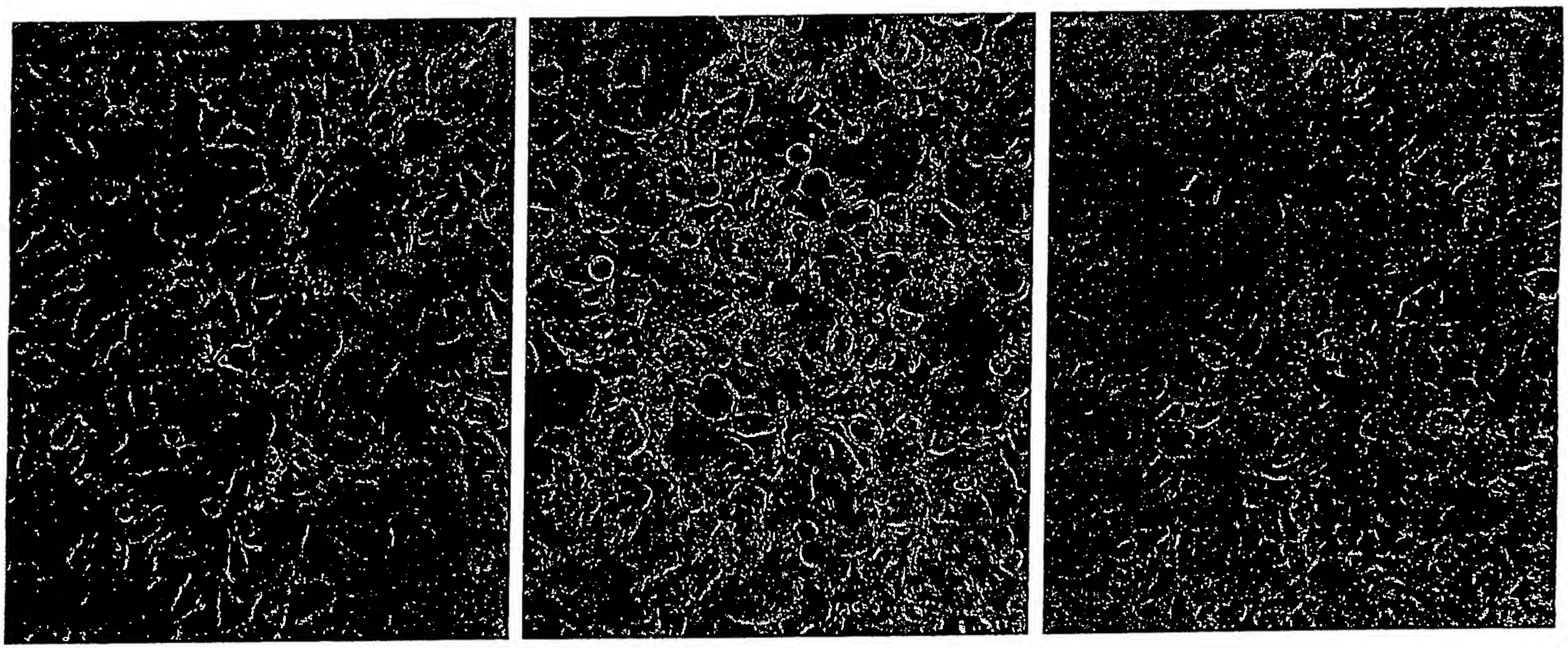
Likewise, other modulators of HIV replication are identified by the inventive screening assays.

CLAIMS

ABSTRACT OF THE DISCLOSURE

The present invention uses gene expression profiling, and gene silencing methods to identify and provide a plurality of 'validated' HIV-induced cellular gene sequences (*e.g.*, HMG20B, HRH1, NP and c-YES) and pathways useful as targets for modulation of HIV-mediated cellular effects. Particular embodiments provide therapeutic compositions, and methods for modulation of HIV infection, replication, or other HIV-related conditions or diseases, comprising inhibition of HIV-induced gene sequences. Additional embodiments provide screening assays for compounds useful to modulate HIV infection, replication, or HIV-related conditions or diseases. Further embodiments provide diagnostic and/or prognostic assays for HIV infection and/or replication.

Figure 1



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Concentrated 89.6
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Figure 2A

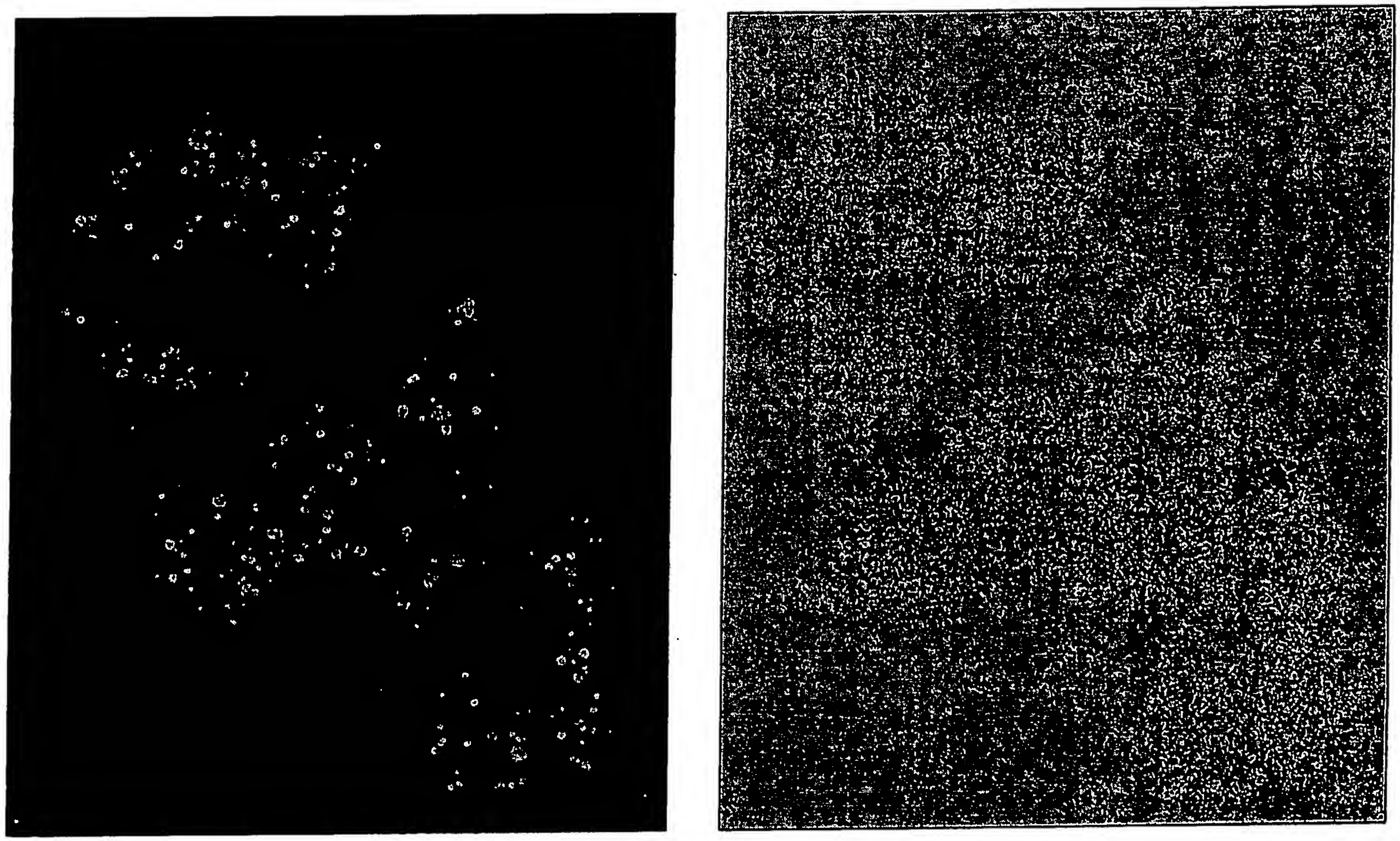

Figure 2B

Figure 3A

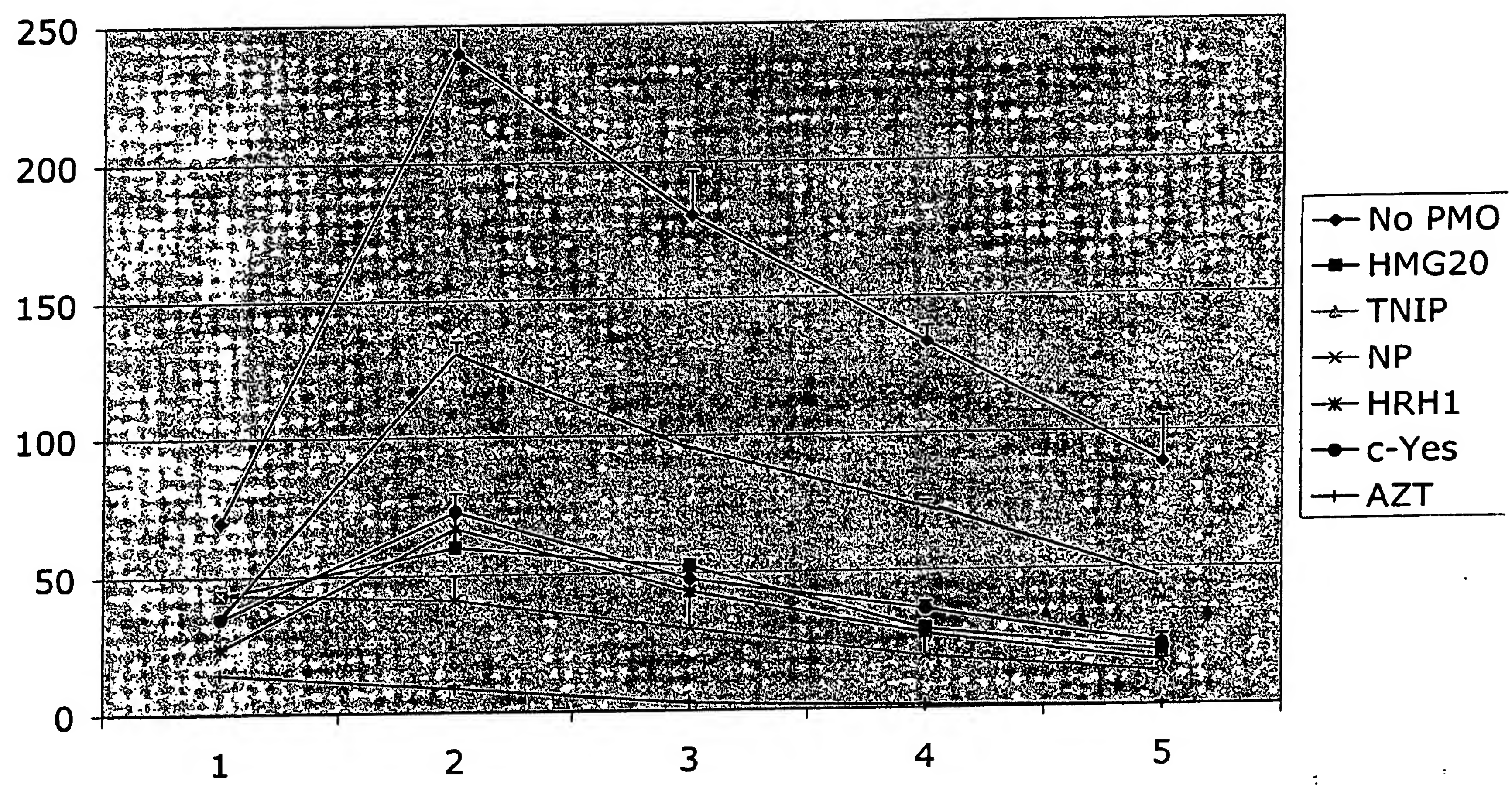

Figure 3B

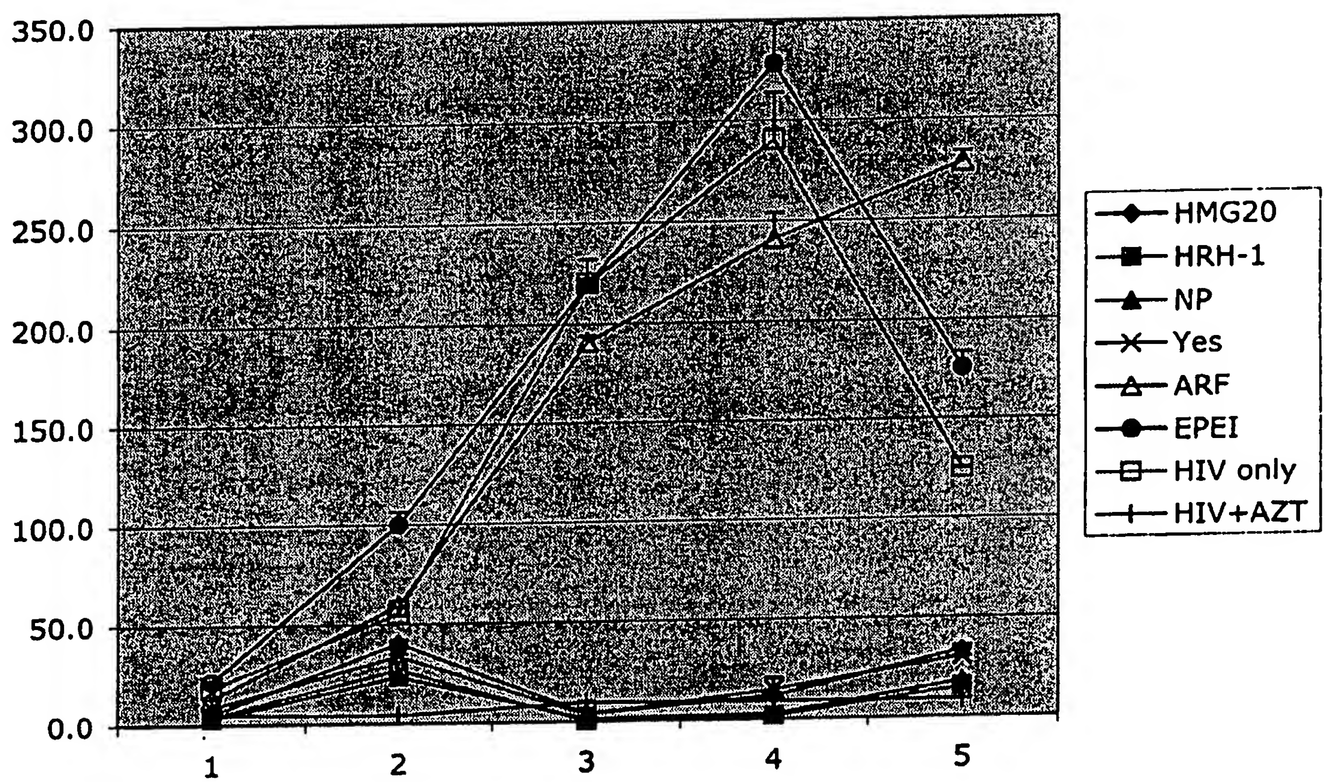
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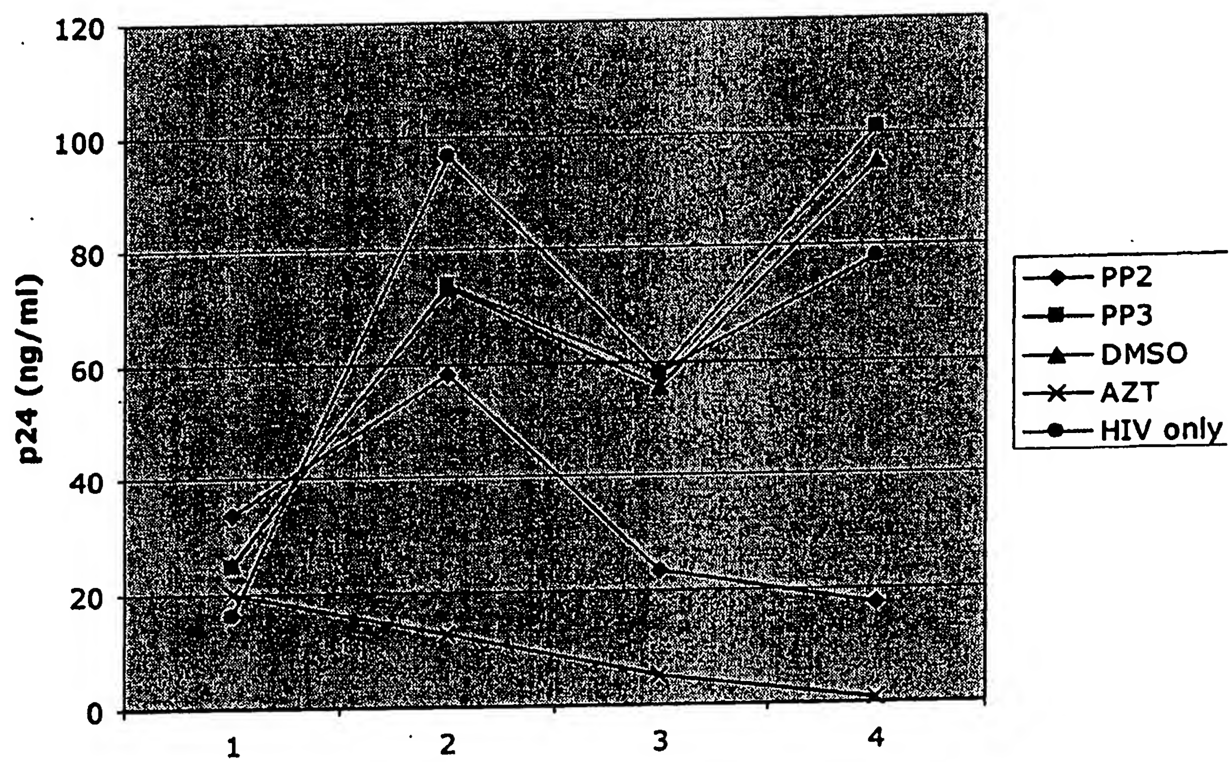
Figure 4



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APPLICATION DATA SHEET

Application Information

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Title ::	METHODS OF TREATMENT AND DIAGNOSIS OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND HIV-RELATED DISEASES
Attorney Docket Number::	49321-106
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Request for Non-Publication?::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	6
Small Entity?::	Yes
Petition included?::	No
Petition Type::	
Licensed U.S. Gov't Agency::	Yes
Contract or Grant No::	NIH/NIAID 1R41 AI055218-01

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Assignee Information

Assignee name::	
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tgc acg gag aag atc cag gag aag aag atc aag aaa gaa gac tcg agc 195
Cys Thr Glu Lys Ile Gln Glu Lys Lys Ile Lys Lys Glu Asp Ser Ser
45 50 55

tct ggg ctc atg aac act ctc ctg aat gga cac aag ggt ggg gac tgc 243
Ser Gly Leu Met Asn Thr Leu Leu Asn Gly His Lys Gly Gly Asp Cys
60 65 70 75

gat ggc ttc tcc acc ttc gat gtt ccc atc ttc act gaa gag ttc ttg 291
Asp Gly Phe Ser Thr Phe Asp Val Pro Ile Phe Thr Glu Glu Phe Leu
80 85 90

gac caa aac aaa gcg cgt gag gcg gag ctt cgg cgc ttg cgg aag atg 339
Asp Gln Asn Lys Ala Arg Glu Ala Glu Leu Arg Arg Leu Arg Lys Met
95 100 105

aat gtg gcc ttc gag gag cag aac gcg gta ctg cag agg caa aac gca 387
Asn Val Ala Phe Glu Glu Gln Asn Ala Val Leu Gln Arg Gln Asn Ala
110 115 120

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gag cat gag cag cgc gcg cga gcg tct gga gca gga gct ggc gct gga      435
Glu His Glu Gln Arg Ala Arg Ala Ser Gly Ala Gly Ala Gly Ala Gly
    125                 130                 135

gga gcg gag gac gct ggc gct gca gca gca gct cca ggc cgt gcg cca      483
Gly Ala Glu Asp Ala Gly Ala Ala Ala Ala Ala Pro Gly Arg Ala Pro
140                 145                 150                 155

ggc gct cac cgc cag ctt cgc ctc act gcc ggt gcc ggg cac ggg cga      531
Gly Ala His Arg Gln Leu Arg Leu Thr Ala Gly Ala Gly His Gly Arg
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aac gcc cac gct ggg cac tct gga ctt cta cat ggc ccg gct tca cgg      579
Asn Ala His Ala Gly His Ser Gly Leu Leu His Gly Pro Ala Ser Arg
            175                 180                 185

agc cat cga gcg cga ccc cgc cca gca cga gaa gct cat cgt ccg cat      627
Ser His Arg Ala Arg Pro Arg Pro Ala Arg Glu Ala His Arg Pro His
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caa gga aat cct ggc cca ggt cgc cag cga gca cct gtg agg agt ggg      675
Gln Gly Asn Pro Gly Pro Gly Arg Gln Arg Ala Pro Val Arg Ser Gly
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cgg gcc cac gat gca gag gag aag ctg tgg gcg cgg ccc tgc cac acc      723
Arg Ala His Asp Ala Glu Glu Lys Leu Trp Ala Arg Pro Cys His Thr
220                 225                 230                 235

cca ccc cgt gga cga gag gct ggg ggt cca ccc ttt ggg gcc tgg tcc      771
Pro Pro Arg Gly Arg Glu Ala Gly Gly Pro Pro Phe Gly Ala Trp Ser
                240                 245                 250

cat cct gca cct ttg ggg gct cca gcc ccc cta aaa tta aat ttc tgc      819
His Pro Ala Pro Leu Gly Ala Pro Ala Pro Leu Lys Leu Asn Phe Cys
            255                 260                 265

agc atc cct tta gct ttc aat ctc ccc agc ccc ctg aac ccg gaa aaa      867
Ser Ile Pro Leu Ala Phe Asn Leu Pro Ser Pro Leu Asn Pro Glu Lys
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gca ctc gct gcg cga tac acc cag aag aac ctc aca gcc gag ggt gcc      915
Ala Leu Ala Ala Arg Tyr Thr Gln Lys Asn Leu Thr Ala Glu Gly Ala
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cct cct cgg agg aca gcc acg cgc tac act ggc tct ccg ggc cac ccc      963
Pro Pro Arg Arg Thr Ala Thr Arg Tyr Thr Gly Ser Pro Gly His Pro
300                 305                 310                 315

cag gac aca ggg cag acg aaa ccc acc ccc agc aca cgg cag gac ccc     1011
Gln Asp Thr Gly Gln Thr Lys Pro Thr Pro Ser Thr Arg Gln Asp Pro
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cca aat tac tca cta cgg ggg gct gtg cca tag gccacacagg aagctgcctt   1064
Pro Asn Tyr Ser Leu Arg Gly Ala Val Pro
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35 40 45

Gln Glu Lys Lys Ile Lys Lys Glu Asp Ser Ser Ser Gly Leu Met Asn
50 55 60

Thr Leu Leu Asn Gly His Lys Gly Gly Asp Cys Asp Gly Phe Ser Thr
65 70 75 80

Phe Asp Val Pro Ile Phe Thr Glu Glu Phe Leu Asp Gln Asn Lys Ala
85 90 95

Arg Glu Ala Glu Leu Arg Arg Leu Arg Lys Met Asn Val Ala Phe Glu
100 105 110

Glu Gln Asn Ala Val Leu Gln Arg Gln Asn Ala Glu His Glu Gln Arg
115 120 125

Ala Arg Ala Ser Gly Ala Gly Ala Gly Ala Gly Gly Ala Glu Asp Ala
130 135 140

Gly Ala Ala Ala Ala Ala Pro Gly Arg Ala Pro Gly Ala His Arg Gln
145 150 155 160

Leu Arg Leu Thr Ala Gly Ala Gly His Gly Arg Asn Ala His Ala Gly
165 170 175

His Ser Gly Leu Leu His Gly Pro Ala Ser Arg Ser His Arg Ala Arg
180 185 190

Pro Arg Pro Ala Arg Glu Ala His Arg Pro His Gln Gly Asn Pro Gly
195 200 205

Pro Gly Arg Gln Arg Ala Pro Val Arg Ser Gly Arg Ala His Asp Ala
210 215 220

Glu Glu Lys Leu Trp Ala Arg Pro Cys His Thr Pro Pro Arg Gly Arg
225 230 235 240

Glu Ala Gly Gly Pro Pro Phe Gly Ala Trp Ser His Pro Ala Pro Leu
245 250 255

Gly Ala Pro Ala Pro Leu Lys Leu Asn Phe Cys Ser Ile Pro Leu Ala
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Phe Asn Leu Pro Ser Pro Leu Asn Pro Glu Lys Ala Leu Ala Ala Arg
275 280 285

Tyr Thr Gln Lys Asn Leu Thr Ala Glu Gly Ala Pro Pro Arg Arg Thr
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atg agc ctc ccc aat tcc tcc tgc ctc tta gaa gac aag atg tgt gag 226

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Met Ser Leu Pro Asn Ser Ser Cys Leu Leu Glu Asp Lys Met Cys Glu
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ggc aac aag acc act atg gcc agc ccc cag ctg atg ccc ctg gtg gtg      274
Gly Asn Lys Thr Thr Met Ala Ser Pro Gln Leu Met Pro Leu Val Val
            20                  25                  30

gtc ctg agc act atc tgc ttg gtc aca gta ggg ctc aac ctg ctg gtg      322
Val Leu Ser Thr Ile Cys Leu Val Thr Val Gly Leu Asn Leu Leu Val
        35                  40                  45

ctg tat gcc gta cgg agt gag cgg aag ctc cac act gtg ggg aac ctg      370
Leu Tyr Ala Val Arg Ser Glu Arg Lys Leu His Thr Val Gly Asn Leu
    50                  55                  60

tac atc gtc agc ctc tcg gtg gcg gac ttg atc gtg ggt gcc gtc gtc      418
Tyr Ile Val Ser Leu Ser Val Ala Asp Leu Ile Val Gly Ala Val Val
65                  70                  75                  80

atg cct atg aac atc ctc tac ctg ctc atg tcc aag tgg tca ctg ggc      466
Met Pro Met Asn Ile Leu Tyr Leu Leu Met Ser Lys Trp Ser Leu Gly
                85                  90                  95

cgt cct ctc tgc ctc ttt tgg ctt tcc atg gac tat gtg gcc agc aca      514
Arg Pro Leu Cys Leu Phe Trp Leu Ser Met Asp Tyr Val Ala Ser Thr
            100                 105                 110

gcg tcc att ttc agt gtc ttc atc ctg tgc att gat cgc tac cgc tct      562
Ala Ser Ile Phe Ser Val Phe Ile Leu Cys Ile Asp Arg Tyr Arg Ser
        115                 120                 125

gtc cag cag ccc ctc agg tac ctt aag tat cgt acc aag acc cga gcc      610
Val Gln Gln Pro Leu Arg Tyr Leu Lys Tyr Arg Thr Lys Thr Arg Ala
    130                 135                 140

tcg gcc acc att ctg ggg gcc tgg ttt ctc tct ttt ctg tgg gtt att      658
Ser Ala Thr Ile Leu Gly Ala Trp Phe Leu Ser Phe Leu Trp Val Ile
145                 150                 155                 160

ccc att cta ggc tgg aat cac ttc atg cag cag acc tcg gtg cgc cga      706
Pro Ile Leu Gly Trp Asn His Phe Met Gln Gln Thr Ser Val Arg Arg
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gag gac aag tgt gag aca gac ttc tat gat gtc acc tgg ttc aag gtc      754
Glu Asp Lys Cys Glu Thr Asp Phe Tyr Asp Val Thr Trp Phe Lys Val
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atg act gcc atc atc aac ttc tac ctg ccc acc ttg ctc atg ctc tgg      802
Met Thr Ala Ile Ile Asn Phe Tyr Leu Pro Thr Leu Leu Met Leu Trp
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ttc tat gcc aag atc tac aag gcc gta cga caa cac tgc cag cac cgg      850
Phe Tyr Ala Lys Ile Tyr Lys Ala Val Arg Gln His Cys Gln His Arg
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gag ctc atc aat agg tcc ctc cct tcc ttc tca gaa att aag ctg agg      898
Glu Leu Ile Asn Arg Ser Leu Pro Ser Phe Ser Glu Ile Lys Leu Arg
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cca gag aac ccc aag ggg gat gcc aag aaa cca ggg aag gag tct ccc      946
Pro Glu Asn Pro Lys Gly Asp Ala Lys Lys Pro Gly Lys Glu Ser Pro
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tgg gag gtt ctg aaa agg aag cca aaa gat gct ggt ggt gga tct gtc      994
Trp Glu Val Leu Lys Arg Lys Pro Lys Asp Ala Gly Gly Gly Ser Val
            260                 265                 270

ttg aag tca cca tcc caa acc ccc aag gag atg aaa tcc cca gtt gtc     1042
Leu Lys Ser Pro Ser Gln Thr Pro Lys Glu Met Lys Ser Pro Val Val
        275                 280                 285

ttc agc caa gag gat gat aga gaa gta gac aaa ctc tac tgc ttt cca     1090
Phe Ser Gln Glu Asp Asp Arg Glu Val Asp Lys Leu Tyr Cys Phe Pro
    290                 295                 300

ctt gat att gtg cac atg cag gct gcg gca gag ggg agt agc agg gac     1138
Leu Asp Ile Val His Met Gln Ala Ala Ala Glu Gly Ser Ser Arg Asp
305                 310                 315                 320

tat gta gcc gtc aac cgg agc cat ggc cag ctc aag aca gat gag cag     1186
Tyr Val Ala Val Asn Arg Ser His Gly Gln Leu Lys Thr Asp Glu Gln
                325                 330                 335

ggc ctg aac aca cat ggg gcc agc gag ata tca gag gat cag atg tta     1234
Gly Leu Asn Thr His Gly Ala Ser Glu Ile Ser Glu Asp Gln Met Leu
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ggt gat agc caa tcc ttc tct cga acg gac tca gat acc acc aca gag     1282
Gly Asp Ser Gln Ser Phe Ser Arg Thr Asp Ser Asp Thr Thr Thr Glu
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aca gca cca ggc aaa ggc aaa ttg agg agt ggg tct aac aca ggc ctg     1330
Thr Ala Pro Gly Lys Gly Lys Leu Arg Ser Gly Ser Asn Thr Gly Leu
    370                 375                 380

gat tac atc aag ttt act tgg aag agg ctc cgc tcg cat tca aga cag     1378
Asp Tyr Ile Lys Phe Thr Trp Lys Arg Leu Arg Ser His Ser Arg Gln
385                 390                 395                 400

tat gta tct ggg ttg cac atg aac cgc gaa agg aag gcc gcc aaa cag     1426
Tyr Val Ser Gly Leu His Met Asn Arg Glu Arg Lys Ala Ala Lys Gln
                405                 410                 415

ttg ggt ttt atc atg gca gcc ttc atc ctc tgc tgg atc cct tat ttc     1474
Leu Gly Phe Ile Met Ala Ala Phe Ile Leu Cys Trp Ile Pro Tyr Phe
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atc ttc ttc atg gtc att gcc ttc tgc aag aac tgt tgc aat gaa cat     1522
Ile Phe Phe Met Val Ile Ala Phe Cys Lys Asn Cys Cys Asn Glu His
        435                 440                 445

ttg cac atg ttc acc atc tgg ctg ggc tac atc aac tcc aca ctg aac     1570
Leu His Met Phe Thr Ile Trp Leu Gly Tyr Ile Asn Ser Thr Leu Asn
    450                 455                 460

ccc ctc atc tac ccc ttg tgc aat gag aac ttc aag aag aca ttc aag     1618
Pro Leu Ile Tyr Pro Leu Cys Asn Glu Asn Phe Lys Lys Thr Phe Lys
465                 470                 475                 480
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aga att ctg cat att cgc tcc taa gggaggctct gaggggatgc aacaaaatga 1672
Arg Ile Leu His Ile Arg Ser
485

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Leu Tyr Ala Val Arg Ser Glu Arg Lys Leu His Thr Val Gly Asn Leu
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Tyr Ile Val Ser Leu Ser Val Ala Asp Leu Ile Val Gly Ala Val Val
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Met Pro Met Asn Ile Leu Tyr Leu Leu Met Ser Lys Trp Ser Leu Gly
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Arg Pro Leu Cys Leu Phe Trp Leu Ser Met Asp Tyr Val Ala Ser Thr
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Ala Ser Ile Phe Ser Val Phe Ile Leu Cys Ile Asp Arg Tyr Arg Ser
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Val Gln Gln Pro Leu Arg Tyr Leu Lys Tyr Arg Thr Lys Thr Arg Ala
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Ser Ala Thr Ile Leu Gly Ala Trp Phe Leu Ser Phe Leu Trp Val Ile
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Pro Ile Leu Gly Trp Asn His Phe Met Gln Gln Thr Ser Val Arg Arg
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Glu Asp Lys Cys Glu Thr Asp Phe Tyr Asp Val Thr Trp Phe Lys Val
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Phe Tyr Ala Lys Ile Tyr Lys Ala Val Arg Gln His Cys Gln His Arg
210 215 220

Glu Leu Ile Asn Arg Ser Leu Pro Ser Phe Ser Glu Ile Lys Leu Arg
225 230 235 240

Pro Glu Asn Pro Lys Gly Asp Ala Lys Lys Pro Gly Lys Glu Ser Pro
245 250 255

Trp Glu Val Leu Lys Arg Lys Pro Lys Asp Ala Gly Gly Gly Ser Val
260 265 270

Leu Lys Ser Pro Ser Gln Thr Pro Lys Glu Met Lys Ser Pro Val Val
275 280 285

Phe Ser Gln Glu Asp Asp Arg Glu Val Asp Lys Leu Tyr Cys Phe Pro
290 295 300

Leu Asp Ile Val His Met Gln Ala Ala Ala Glu Gly Ser Ser Arg Asp
305 310 315 320

Tyr Val Ala Val Asn Arg Ser His Gly Gln Leu Lys Thr Asp Glu Gln
325 330 335

Gly Leu Asn Thr His Gly Ala Ser Glu Ile Ser Glu Asp Gln Met Leu
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Gly Asp Ser Gln Ser Phe Ser Arg Thr Asp Ser Asp Thr Thr Thr Glu
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Thr Ala Pro Gly Lys Gly Lys Leu Arg Ser Gly Ser Asn Thr Gly Leu
370 375 380

Asp Tyr Ile Lys Phe Thr Trp Lys Arg Leu Arg Ser His Ser Arg Gln
385 390 395 400

Tyr Val Ser Gly Leu His Met Asn Arg Glu Arg Lys Ala Ala Lys Gln
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Leu Gly Phe Ile Met Ala Ala Phe Ile Leu Cys Trp Ile Pro Tyr Phe
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Ile Phe Phe Met Val Ile Ala Phe Cys Lys Asn Cys Cys Asn Glu His
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Leu His Met Phe Thr Ile Trp Leu Gly Tyr Ile Asn Ser Thr Leu Asn
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tctgttgtgt ttgtcaaaaa gtcattgtaa tctttcatag ccatacctgg taagcaaaaa 3240

ctagtaaaga cataggaaca tgcagtttta cttggtgttt atgttgcaat ctggttgtga 3300

tttatatttt aaagcttggt gctaaaccac aatatgtata gcacatggag tgcctgtaca 3360

agctgatgtt ttgtattttg tgttcctctt tgcatgatct gtcaaagtga gatattttta 3420

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Met Glu Asn
1

gga tac acc tat gaa gat tat aag aac act gca gaa tgg ctt ctg tct 166
Gly Tyr Thr Tyr Glu Asp Tyr Lys Asn Thr Ala Glu Trp Leu Leu Ser
5 10 15

cat act aag cac cga cct caa gtt gca ata atc tgt ggt tct gga tta 214
His Thr Lys His Arg Pro Gln Val Ala Ile Ile Cys Gly Ser Gly Leu
20 25 30 35

gga ggt ctg act gat aaa tta act cag gcc cag atc ttt gac tac agt 262
Gly Gly Leu Thr Asp Lys Leu Thr Gln Ala Gln Ile Phe Asp Tyr Ser
40 45 50

gaa atc ccc aac ttt cct cga agt aca gtg cca ggt cat gct ggc cga 310
Glu Ile Pro Asn Phe Pro Arg Ser Thr Val Pro Gly His Ala Gly Arg
55 60 65

ctg gtg ttt ggg ttc ctg aat ggc agg gcc tgt gtg atg atg cag ggc 358
Leu Val Phe Gly Phe Leu Asn Gly Arg Ala Cys Val Met Met Gln Gly
70 75 80

agg ttc cac atg tat gaa ggg tac cca ctc tgg aag gtg aca ttc cca 406
Arg Phe His Met Tyr Glu Gly Tyr Pro Leu Trp Lys Val Thr Phe Pro
85 90 95

gtg agg gtt ttc cac ctt ctg ggt gtg gac acc ctg gta gtc acc aat 454
Val Arg Val Phe His Leu Leu Gly Val Asp Thr Leu Val Val Thr Asn
100 105 110 115

gca gca gga ggg ctg aac ccc aag ttt gag gtt gga gat atc atg ctg 502
Ala Ala Gly Gly Leu Asn Pro Lys Phe Glu Val Gly Asp Ile Met Leu
120 125 130

atc cgt gac cat atc aac cta cct ggt ttc agt ggt cag aac cct ctc 550
Ile Arg Asp His Ile Asn Leu Pro Gly Phe Ser Gly Gln Asn Pro Leu
135 140 145

aga ggg ccc aat gat gaa agg ttt gga gat cgt ttc cct gcc atg tct 598
Arg Gly Pro Asn Asp Glu Arg Phe Gly Asp Arg Phe Pro Ala Met Ser
150 155 160

gat gcc tac gac cgg act atg agg cag agg gct ctc agt acc tgg aaa 646
Asp Ala Tyr Asp Arg Thr Met Arg Gln Arg Ala Leu Ser Thr Trp Lys
165 170 175

caa atg ggg gag caa cgt gag cta cag gaa ggc acc tat gtg atg gtg 694
Gln Met Gly Glu Gln Arg Glu Leu Gln Glu Gly Thr Tyr Val Met Val
180 185 190 195

gca ggc ccc agc ttt gag act gtg gca gaa tgt cgt gtg ctg cag aag 742
Ala Gly Pro Ser Phe Glu Thr Val Ala Glu Cys Arg Val Leu Gln Lys
200 205 210

ctg gga gca gac gct gtt ggc atg agt aca gta cca gaa gtt atc gtt 790
Leu Gly Ala Asp Ala Val Gly Met Ser Thr Val Pro Glu Val Ile Val
215 220 225

gca cgg cac tgt gga ctt cga gtc ttt ggc ttc tca ctc atc act aac 838
Ala Arg His Cys Gly Leu Arg Val Phe Gly Phe Ser Leu Ile Thr Asn
230 235 240

aag gtc atc atg gat tat gaa agc ctg gag aag gcc aac cat gaa gaa 886
Lys Val Ile Met Asp Tyr Glu Ser Leu Glu Lys Ala Asn His Glu Glu

245 250 255

gtc tta gca gct ggc aaa caa gct gca cag aaa ttg gaa cag ttt gtc 934
Val Leu Ala Ala Gly Lys Gln Ala Ala Gln Lys Leu Glu Gln Phe Val
260 265 270 275

tcc att ctt atg gcc agc att cca ctc cct gac aaa gcc agt tga 979
Ser Ile Leu Met Ala Ser Ile Pro Leu Pro Asp Lys Ala Ser
280 285

cctgccttgg agtcgtctgg catctcccac acaagaccca agtagctgct accttctttg 1039

gccccttgct ggagtcatgt gcctctgtcc ttaggttgta gcagaaagga aaagattcct 1099

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<212> PRT
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20 25 30

Ser Gly Leu Gly Gly Leu Thr Asp Lys Leu Thr Gln Ala Gln Ile Phe
35 40 45

Asp Tyr Ser Glu Ile Pro Asn Phe Pro Arg Ser Thr Val Pro Gly His
50 55 60

Ala Gly Arg Leu Val Phe Gly Phe Leu Asn Gly Arg Ala Cys Val Met
65 70 75 80

Met Gln Gly Arg Phe His Met Tyr Glu Gly Tyr Pro Leu Trp Lys Val
85 90 95

Thr Phe Pro Val Arg Val Phe His Leu Leu Gly Val Asp Thr Leu Val

            100   105   110

Val Thr Asn Ala Ala Gly Gly Leu Asn Pro Lys Phe Glu Val Gly Asp
        115   120   125

Ile Met Leu Ile Arg Asp His Ile Asn Leu Pro Gly Phe Ser Gly Gln
    130   135   140

Asn Pro Leu Arg Gly Pro Asn Asp Glu Arg Phe Gly Asp Arg Phe Pro
145   150   155   160

Ala Met Ser Asp Ala Tyr Asp Arg Thr Met Arg Gln Arg Ala Leu Ser
                165   170   175

Thr Trp Lys Gln Met Gly Glu Gln Arg Glu Leu Gln Glu Gly Thr Tyr
            180   185   190

Val Met Val Ala Gly Pro Ser Phe Glu Thr Val Ala Glu Cys Arg Val
        195   200   205

Leu Gln Lys Leu Gly Ala Asp Ala Val Gly Met Ser Thr Val Pro Glu
    210   215   220

Val Ile Val Ala Arg His Cys Gly Leu Arg Val Phe Gly Phe Ser Leu
225   230   235   240

Ile Thr Asn Lys Val Ile Met Asp Tyr Glu Ser Leu Glu Lys Ala Asn
                245   250   255

His Glu Glu Val Leu Ala Ala Gly Lys Gln Ala Ala Gln Lys Leu Glu
            260   265   270

Gln Phe Val Ser Ile Leu Met Ala Ser Ile Pro Leu Pro Asp Lys Ala
        275   280   285

Ser

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           1               5                   10

att aaa tac aga cct gaa aat act cca gag cct gtc agt aca agt gtg       97
Ile Lys Tyr Arg Pro Glu Asn Thr Pro Glu Pro Val Ser Thr Ser Val
    15                  20                  25

agc cat tat gga gca gaa ccc act aca gtg tca cca tgt ccg tca tct      145
Ser His Tyr Gly Ala Glu Pro Thr Thr Val Ser Pro Cys Pro Ser Ser
30                  35                  40                  45

tca gca aag gga aca gca gtt aat ttc agc agt ctt tcc atg aca cca      193
Ser Ala Lys Gly Thr Ala Val Asn Phe Ser Ser Leu Ser Met Thr Pro
                50                  55                  60

ttt gga gga tcc tca ggg gta acg cct ttt gga ggt gca tct tcc tca      241
Phe Gly Gly Ser Ser Gly Val Thr Pro Phe Gly Gly Ala Ser Ser Ser
            65                  70                  75

ttt tca gtg gtg cca agt tca tat cct gct ggt tta aca ggt ggt gtt      289
Phe Ser Val Val Pro Ser Ser Tyr Pro Ala Gly Leu Thr Gly Gly Val
        80                  85                  90

act ata ttt gtg gcc tta tat gat tat gaa gct aga act aca gaa gac      337
Thr Ile Phe Val Ala Leu Tyr Asp Tyr Glu Ala Arg Thr Thr Glu Asp
    95                  100                 105

ctt tca ttt aag aag ggt gaa aga ttt caa ata att aac aat acg gaa      385
Leu Ser Phe Lys Lys Gly Glu Arg Phe Gln Ile Ile Asn Asn Thr Glu
110                 115                 120                 125

gga gat tgg tgg gaa gca aga tca atc gct aca gga aag aat ggt tat      433
Gly Asp Trp Trp Glu Ala Arg Ser Ile Ala Thr Gly Lys Asn Gly Tyr
                130                 135                 140

atc ccg agc aat tat gta gcg cct gca gat tcc att cag gca gaa gaa      481
Ile Pro Ser Asn Tyr Val Ala Pro Ala Asp Ser Ile Gln Ala Glu Glu
            145                 150                 155

tgg tat ttt ggc aaa atg ggg aga aaa gat gct gaa aga tta ctt ttg      529
Trp Tyr Phe Gly Lys Met Gly Arg Lys Asp Ala Glu Arg Leu Leu Leu
        160                 165                 170

aat cct gga aat caa cga ggt att ttc tta gta aga gag agt gaa aca      577
Asn Pro Gly Asn Gln Arg Gly Ile Phe Leu Val Arg Glu Ser Glu Thr
    175                 180                 185

act aaa ggt gct tat tcc ctt tct att cgt gat tgg gat gag ata agg      625
Thr Lys Gly Ala Tyr Ser Leu Ser Ile Arg Asp Trp Asp Glu Ile Arg
190                 195                 200                 205

ggt gac aat gtg aaa cac tac aaa att agg aaa ctt gac aat ggt gga      673
Gly Asp Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp Asn Gly Gly
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tac tat atc aca acc aga gca caa ttt gat act ctg cag aaa ttg gtg      721
Tyr Tyr Ile Thr Thr Arg Ala Gln Phe Asp Thr Leu Gln Lys Leu Val
        225                 230                 235

aaa cac tac aca gaa cat gct gat ggt tta tgc cac aag ttg aca act      769
Lys His Tyr Thr Glu His Ala Asp Gly Leu Cys His Lys Leu Thr Thr
    240                 245                 250

gtg tgt cca act gtg aaa cct cag act caa ggt cta gca aaa gat gct      817
Val Cys Pro Thr Val Lys Pro Gln Thr Gln Gly Leu Ala Lys Asp Ala
255                 260                 265

tgg gaa atc cct cga gaa tct ttg cga cta gag gtt aaa cta gga caa      865
Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys Leu Gly Gln
270                 275                 280                 285

gga tgt ttc ggc gaa gtg tgg atg gga aca tgg aat gga acc acg aaa      913
Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly Thr Thr Lys
                290                 295                 300

gta gca atc aaa aca cta aaa cca ggt aca atg atg cca gaa gct ttc      961
Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Met Pro Glu Ala Phe
            305                 310                 315

ctt caa gaa gct cag ata atg aaa aaa tta aga cat gat aaa ctt gtt     1009
Leu Gln Glu Ala Gln Ile Met Lys Lys Leu Arg His Asp Lys Leu Val
        320                 325                 330

cca cta tat gct gtt gtt tct gaa gaa cca att tac att gtc act gaa     1057
Pro Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile Val Thr Glu
    335                 340                 345

ttt atg tca aaa gga agc tta tta gat ttc ctt aag gaa gga gat gga     1105
Phe Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Glu Gly Asp Gly
350                 355                 360                 365

aag tat ttg aag ctt cca cag ctg gtt gat atg gct gct cag att gct     1153
Lys Tyr Leu Lys Leu Pro Gln Leu Val Asp Met Ala Ala Gln Ile Ala
                370                 375                 380

gat ggt atg gca tat att gaa aga atg aac tat att cac cga gat ctt     1201
Asp Gly Met Ala Tyr Ile Glu Arg Met Asn Tyr Ile His Arg Asp Leu
            385                 390                 395

cgg gct gct aat att ctt gta gga gaa aat ctt gtg tgc aaa ata gca     1249
Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys Lys Ile Ala
        400                 405                 410

gac ttt ggt tta gca agg tta att gaa gac aat gaa tac aca gca aga     1297
Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr Thr Ala Arg
    415                 420                 425

caa ggt gca aaa ttt cca atc aaa tgg aca gct cct gaa gct gca ctg     1345
Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu Ala Ala Leu
430                 435                 440                 445

tat ggt cgg ttt aca ata aag tct gat gtc tgg tca ttt gga att ctg     1393
Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe Gly Ile Leu
                450                 455                 460
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caa aca gaa cta gta aca aag ggc cga gtg cca tat cca ggt atg gtg      1441
Gln Thr Glu Leu Val Thr Lys Gly Arg Val Pro Tyr Pro Gly Met Val
            465                 470                 475

aac cgt gaa gta cta gaa caa gtg gag cga gga tac agg atg ccg tgc      1489
Asn Arg Glu Val Leu Glu Gln Val Glu Arg Gly Tyr Arg Met Pro Cys
        480                 485                 490

cct cag ggc tgt cca gaa tcc ctc cat gaa ttg atg aat ctg tgt tgg      1537
Pro Gln Gly Cys Pro Glu Ser Leu His Glu Leu Met Asn Leu Cys Trp
    495                 500                 505

aag aag gac cct gat gaa aga cca aca ttt gaa tat att cag tcc ttc      1585
Lys Lys Asp Pro Asp Glu Arg Pro Thr Phe Glu Tyr Ile Gln Ser Phe
510                 515                 520                 525

ttg gaa gac tac ttc act gct aca gag cca cag tac cag cca gga gaa      1633
Leu Glu Asp Tyr Phe Thr Ala Thr Glu Pro Gln Tyr Gln Pro Gly Glu
                530                 535                 540

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Asn Leu


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aagtattaaa ctctaatgta ccaatgatga atttatcagc gtatttcagg gtccaaacaa    1862

aatagagcta agatactgat gacagtgtgg gtgacagcat ggtaatgaag gacagtgagg    1922

ctcctgctta tttataaatc atttcctttc ttttttccc caaagtcaga attgctcaaa     1982

gaaaattatt tattgttaca gataaaactt gagagataaa aagctatacc ataataaaat    2042

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        35   40   45

Gly Thr Ala Val Asn Phe Ser Ser Leu Ser Met Thr Pro Phe Gly Gly
    50   55   60

Ser Ser Gly Val Thr Pro Phe Gly Gly Ala Ser Ser Ser Phe Ser Val
65   70   75   80

Val Pro Ser Ser Tyr Pro Ala Gly Leu Thr Gly Gly Val Thr Ile Phe
                85   90   95

Val Ala Leu Tyr Asp Tyr Glu Ala Arg Thr Thr Glu Asp Leu Ser Phe
            100   105   110

Lys Lys Gly Glu Arg Phe Gln Ile Ile Asn Asn Thr Glu Gly Asp Trp
        115   120   125

Trp Glu Ala Arg Ser Ile Ala Thr Gly Lys Asn Gly Tyr Ile Pro Ser
    130   135   140

Asn Tyr Val Ala Pro Ala Asp Ser Ile Gln Ala Glu Glu Trp Tyr Phe
145   150   155   160

Gly Lys Met Gly Arg Lys Asp Ala Glu Arg Leu Leu Leu Asn Pro Gly
                165   170   175

Asn Gln Arg Gly Ile Phe Leu Val Arg Glu Ser Glu Thr Thr Lys Gly
            180   185   190

Ala Tyr Ser Leu Ser Ile Arg Asp Trp Asp Glu Ile Arg Gly Asp Asn
        195   200   205

Val Lys His Tyr Lys Ile Arg Lys Leu Asp Asn Gly Gly Tyr Tyr Ile
    210   215   220

Thr Thr Arg Ala Gln Phe Asp Thr Leu Gln Lys Leu Val Lys His Tyr
225   230   235   240

Thr Glu His Ala Asp Gly Leu Cys His Lys Leu Thr Thr Val Cys Pro
                245   250   255

Thr Val Lys Pro Gln Thr Gln Gly Leu Ala Lys Asp Ala Trp Glu Ile
            260   265   270

Pro Arg Glu Ser Leu Arg Leu Glu Val Lys Leu Gly Gln Gly Cys Phe
        275   280   285

Gly Glu Val Trp Met Gly Thr Trp Asn Gly Thr Thr Lys Val Ala Ile
    290   295   300

Lys Thr Leu Lys Pro Gly Thr Met Met Pro Glu Ala Phe Leu Gln Glu
305   310   315   320

Ala Gln Ile Met Lys Lys Leu Arg His Asp Lys Leu Val Pro Leu Tyr
                325   330   335

Ala Val Val Ser Glu Glu Pro Ile Tyr Ile Val Thr Glu Phe Met Ser
            340   345   350

Lys Gly Ser Leu Leu Asp Phe Leu Lys Glu Gly Asp Gly Lys Tyr Leu
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Lys Leu Pro Gln Leu Val Asp Met Ala Ala Gln Ile Ala Asp Gly Met
    370   375   380

Ala Tyr Ile Glu Arg Met Asn Tyr Ile His Arg Asp Leu Arg Ala Ala
385   390   395   400

Asn Ile Leu Val Gly Glu Asn Leu Val Cys Lys Ile Ala Asp Phe Gly
                405   410   415

Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr Thr Ala Arg Gln Gly Ala
            420   425   430

Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu Ala Ala Leu Tyr Gly Arg
        435   440   445

Phe Thr Ile Lys Ser Asp Val Trp Ser Phe Gly Ile Leu Gln Thr Glu

450 455 460

Leu Val Thr Lys Gly Arg Val Pro Tyr Pro Gly Met Val Asn Arg Glu
465 470 475 480

Val Leu Glu Gln Val Glu Arg Gly Tyr Arg Met Pro Cys Pro Gln Gly
485 490 495

Cys Pro Glu Ser Leu His Glu Leu Met Asn Leu Cys Trp Lys Lys Asp
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Pro Asp Glu Arg Pro Thr Phe Glu Tyr Ile Gln Ser Phe Leu Glu Asp
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